

University of St Andrews



Full metadata for this thesis is available in
St Andrews Research Repository
at:

<http://research-repository.st-andrews.ac.uk/>

This thesis is protected by original copyright

Activities of RNA helicase Xp54 in translational control
during *Xenopus* oogenesis

Andrew John Weston

Bute Medical Buildings, School of Biology, University of St. Andrews



Submitted for the degree of degree of Ph.D.
May 2004

Th E769

Declarations

I, Andrew Weston, hereby certify that this thesis, which is approximately 51,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 10/12/04

Signature of candidate.

I was admitted as a research student in September 2000 and as a candidate for the degree of PhD in September 2001; the higher study for which this is a record was carried out in the University of St. Andrews between 2000 and 2003.

Date 10/12/04

Signature of candidate.

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any *bona fide* library or research worker.

Date 10/12/04

Signature of candidate..

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date 17/12/04

Signature of supervisor

Acknowledgements

I would like to thank Dr. John Sommerville for guidance, advice and support in the completion of this project and Dr. David Smillie for practical help and encouragement. Also I would like to thank Dr. Graham Kemp and Professor Keith Sillar for advice and encouragement.

Table of contents

	Page number
Acknowledgements	i
Table of Contents	ii
Index of Figures	v
Abstract	viii
Introduction	1
Maternal mRNA	1
Oocyte maturation	4
Export of mRNA from the nucleus	17
Role of splicing components in the stability of exported mRNA	26
Stability of mRNA in the cytoplasm	35
Importance of RNA secondary structure	46
The protein components of maternal mRNP in <i>Xenopus</i>	55
Masking and unmasking of maternal mRNA	75
Objectives	79
Methods and Materials	81
Media and storage	81
Bacterial Strains	82
Oocyte preparation, injection and extraction for SDS-PAGE	82
Oocyte preparation	82
Oocyte injection	83
Oocyte nucleus and cytoplasm extraction	84
Preparation of oocyte extracts for SDS-PAGE	85
Preparation of Poly(A) structures from oocytes	86
Trichloroacetic acid (TCA) precipitation of radiolabelled oocyte proteins	87
SDS-PAGE, Coomassie staining, electroblotting and immunodetection	88
Stripping of nitrocellulose membranes	92
Preparation of Immunoglobulin G molecules from serum	93
Immunoprecipitation of mRNP	94
Photocrosslinking of mRNP complexes	95
Agarose gel electrophoresis	96
Restriction enzyme digestion of DNA	97
Ligation of DNA	98

DNA extraction from agarose gels	98
Plasmid mini-prep.	99
Transformation by heat shock	101
RNA purification from oocytes by phenol-chloroform extraction	102
Polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR)	103
Construction of expression vectors	105
DNA sequencing and protein sequence analysis and alignment	108
Results	111
The steady-state levels of different mRNAs change throughout the course of oogenesis and have different stabilities	111
mRNAs differ in the degree of their association with Xp54-containing structures	120
Overexpression of Xp54 differentially enhances nuclear export of mRNAs	124
Overexpression of Xp54 selectively destabilises and stabilises different mRNAs	139
An <i>Xp54</i> antisense morpholino inhibits <i>Xp54</i> mRNA translation and affects its stability	144
Injection of the <i>Xp54</i> antisense morpholino affects stability of different mRNAs in different ways	157
Injection of the <i>Xp54</i> antisense morpholino into progesterone-treated oocytes increases the stability of those mRNAs translated only during maturation	172
An antisense morpholino directed against the mRNA masking protein FRGY2 affects the stability of mRNAs depending on translational status	182
Xp54 inhibits and FRGY2 promotes general oocyte mRNA translation	189
Interaction of Xp54 and RAP55 within mRNP particles	200
Discussion	220
The steady-state levels of mRNAs during oogenesis	221
Xp54's influence on the nuclear export of mRNA	225
Xp54 and stability of mRNA	233
•Overexpression of Xp54	233
•Underexpression of Xp54	239
•Underexpression of Xp54 during oocyte maturation	255
Interaction of other mRNP proteins with mRNA and Xp54	261
•Xp54 and FRGY2 as multifunctional proteins	269
•RAP55	272
Summary	279
Further work	284

Appendix A	288
Appendix B	289
Appendix C	295
Appendix D	305
References	307

Index of Figures

	Page number
Figure 1 Sequence of changes occurring during <i>Xenopus</i> oogenesis and maturation.	7
Figure 2 Changes in Mos and MPF activity during oogenesis meiosis and mitosis of early embryogenesis.	14
Figure 3 The leucine-rich putative NES in Xp54 and homology with the HIV REV protein.	21
Figure 4 The series of soluble factors operating to deliver an mRNA molecule through the NPC to the cytoplasm for translation.	23
Figure 5 NMMD-associated mRNA remodelling in mammalian cells and <i>Xenopus</i> oocytes.	31
Figure 6 The mRNA surveillance process in a typical mammalian intron-containing gene.	34
Figure 7 Deadenylation and decapping of mRNA.	38
Figure 8 Stem-and-loop secondary structure formed by pairing between complementary bases in a single strand.	47
Figure 9 Structural models of the third RNA-recognition motif of nucleolin and the cold-shock domain of FRGY2.	60
Figure 10 Structure of FRGY2.	63
Figure 11 Alignment of the DEAD-box RNA helicase subfamily including p54.	66
Figure 12 Position of putative CKII phosphorylation sites within the amino acid sequence at the C-terminus of Xp54.	78
Figure 13 Construction of T7-tagged <i>Xp54</i> expression vector.	107
Figure 14 Oocytes accumulate mRNAs of a wide range of types through oogenesis.	114

Figure 15	Stabilities of oocyte mRNAs differ according to the gene of origin.	118
Figure 16	mRNAs differ in the degree of their association with Xp54-containing structures.	122
Figure 17	Nucleocytoplasmic distribution of recombinant forms of Xp54 expressed from plasmid vectors injected into the nucleus is disrupted by the DQAD mutation.	127
Figure 18	Expression of T7-Xp54 adds to the steady-state levels of oocyte Xp54.	130
Figure 19	RNA gel and quantitative RNA gels for <i>Xp54</i> DNA-injected oocyte samples.	133
Figure 20	(A) Overexpression of Xp54 enhances nuclear export of selected mRNAs (<i>rpL1</i> , <i>rpS1</i> , <i>cyclin B1</i>). (B) Overexpression of wild-type Xp54 tends to destabilise those mRNAs translated during oogenesis (<i>rpL1</i> , <i>rpS1</i>) and stabilise those stored until after oocyte maturation (<i>cyclin B1</i>) relative to overexpression of mutant Xp54.	136 136
Figure 21	Overexpression of wild-type Xp54 tends to destabilise those mRNAs translated during oogenesis (<i>histone H4</i>) and stabilise those stored until after oocyte maturation (<i>FRGY1</i>) relative to overexpression of mutant Xp54.	141
Figure 22	<i>Xp54</i> Morpholino antisense oligonucleotide.	146
Figure 23	An antisense <i>Xp54</i> morpholino inhibits <i>Xp54</i> mRNA translation and affects its mRNA stability.	147-152
Figure 24	Injection of the <i>Xp54</i> antisense morpholino affects stability of mRNAs of different genes over time in different ways.	161

Figure 25	as fig.24	163
Figure 26	Exogenous Xp54 expression does not counteract the stabilisation of <i>rpS1</i> mRNA by the antisense morpholino.	168
Figure 27	The antisense morpholino delays and inhibits oocyte maturation.	173
Figure 28	Injection of the <i>Xp54</i> antisense morpholino into progesterone-treated oocytes increases the stability of mRNAs only translated following maturation onset.	176
Figure 29	Injection of a <i>FRGY2</i> antisense morpholino into oocytes differentially affects the stability of mRNAs.	184
Figure 30	³⁵ S methionine incorporation into proteins is enhanced by inhibition of Xp54 translation and reduced by inhibition of <i>FRGY2</i> translation.	191
Figure 31	Inhibition of Xp54 translation enhances total protein synthesis and inhibition of <i>FRGY2</i> reduces total protein synthesis.	194
Figure 32	New protein synthesis is enhanced by inhibition of Xp54 translation and is reduced by inhibition of <i>FRGY2</i> translation.	198
Figure 33	Xp54 and p52 form coincident complexes following UV-crosslinking treatment.	202
Figure 34	Xp54 forms complexes with overexpressed T7-Xp54 and possibly RAP55 that are susceptible to crosslinking.	206
Figure 35	The presence of RNA promotes crosslinking of p52 to unidentified factors.	209
Figure 36	Consensus regions of XRAPA protein sequence.	218

Abstract

The *Xenopus* mRNP particle component Xp54, an ATP-dependent RNA helicase with metazoan and yeast orthologues, associates with maternally transcribed mRNAs including those encoding proteins for meiosis progression. Xp54's role in nuclear export of such mRNAs and the effect of Xp54 and FRGY2, a cooperating protein, on mRNA stability was examined. The identity and potential interactions with Xp54 of two other mRNP proteins, p52 and RAP55, was also investigated.

The DEAD motif in Xp54 was found to promote its efficient nuclear import. There was a differential nature of interaction of Xp54 with particular mRNAs occasionally depending on when the mRNA is translated — during or after oogenesis.

Functionally disparate mRNAs accumulate gradually during oogenesis. In late oogenesis, certain mRNAs apparently require ongoing transcription for continued presence rather than having a residual storage but were still capable of associating with Xp54.

Overexpressing Xp54 enhanced nuclear export of many mRNAs although their total stability in the oocyte may depend on their translational status and therefore on factors including cytoplasmic fate of the mRNA.

Anti-*Xp54* antisense morpholino microinjection reduced endogenous Xp54 expression and influenced specific mRNA stabilities differently and according to period of oogenesis. This could reflect Xp54's role both in storing mRNA as mRNP and possibly as a translation initiator of non-masked mRNA before maturation. Also, oocyte maturation was significantly delayed or blocked by this Xp54 reduction. Antisense-impeded FRGY2 translation reduction also differentially affected mRNA stability according to their translational status. However, the anti-*Xp54* and anti-*FRGY2* morpholinos promoted and inhibited translation of a wide range of abundant proteins respectively suggesting the major roles of Xp54 and FRGY2.

By sequencing of *Xenopus* cDNA clones similar to *RAP55* cDNA, a likely orthologue was identified with similar motif presence and layout. This is distinct from p52 but evidence for Xp54 interaction is indirect.

Introduction

Maternal RNA

A common feature of early vertebrate development is the accumulation of stored messenger RNA (mRNA) containing the genetic information necessary for subsequent protein production when access to the chromosomes for further mRNA production by transcription is not feasible. In particular this can be a result of the rapid cell divisions occurring during early embryogenesis when the repeated and frequent expansion and condensation of chromatin can effectively preclude gene access (Almouzni and Wolffe, 1993).

The mechanisms of maintaining mRNA in a non-translated state are conserved to varying extents across a range of eukaryotic organisms. The translation repression is achieved commonly by *masking* proteins which bind to the mRNA molecules preventing translation until production of the relevant protein is needed. The resulting structure is an mRNP (messenger ribonucleoprotein) particle. These structures are most commonly associated with oocytes, spermatocytes and embryos. Examples include the amphibian *Xenopus*, where the messages of many genes are stored as mRNP particles in vast quantities during oogenesis (Spirin, 1994); the

nematode *Caenorhabditis elegans* where the masking protein LIN-28 regulates translation in the embryo (Moss *et al.*, 1997) and in mice with the protein MSY2 masking mRNA in spermatids and oocytes (Gu *et al.*, 1998). Also in the fruit fly *Drosophila melanogaster*, the mRNAs encoding for proteins which establish polarity in the embryo are transported to the oocyte in the fly's egg chamber, from the surrounding nurse cells, in the form of mRNP. This association confers the repression of translation of the mRNA until it is localised in the appropriate site within the *Drosophila* oocyte and the mRNP particle consists of proteins including Exuperantia and the RNA helicase Me31B (Nakamura *et al.*, 2001).

The accumulation of mRNA during oogenesis across many eukaryotes is a result of high transcriptional activity which is replaced by high translation rates at the onset of embryogenesis. However, high rates of transcription are also achieved during mammalian spermatogenesis which comes to a halt as the final cellular stage, spermatids, differentiate. Again mRNA is stored during the (usually much shorter) development to be translated at the terminal stages (Kleene, 1996) thereby highlighting similarities to maternal mRNA information storage during gametogenesis in the female. Another important structure with apparent counterparts in other eukaryotes

is the P-granule. This is an mRNA-protein structure found specifically in the germline cells of *C.elegans* (Pitt *et al.*, 2000) and is essential for function of sperm and oocytes. Similar structures exist in *Drosophila* called polar granules which determine the development of germ cells in the embryo (Rongo *et al.*, 1997).

In *Xenopus laevis*, the South African clawed frog, transcription only resumes after the embryonic stage known as mid-blastula transition, having been suspended after fertilisation. The early embryo is thus dependent on the large stores of messenger RNAs within mRNP particles becoming activated to establish the asymmetrical protein expression gradients which drive early development.

Thus it can be seen that amongst widely different eukaryotes there is a common need to store mRNA as mRNP for use at a later time, significantly, embryogenesis. This storage of information has the advantage that the cell can respond immediately by gene expression to an external stimulus, most notably fertilisation, and also that the cell can continue to grow (for example as part of an embryo) when transcription is minimal or absent (Sommerville, 1999).

Oocyte maturation

As described above the major period of mRNP accumulation in most well studied metazoans is during gametogenesis, particularly oogenesis. The terminal stage of oogenesis in most animals is oocyte maturation. This is a well studied and characterised process especially in *Xenopus* although vertebrate development is well conserved during oocyte maturation, fertilisation and early embryogenesis.

After the cleavage of the fertilised oocyte into the eventually hollow blastula, the epithelia of the developing embryo undergo stereotypical movements during a phase called gastrulation and proceeds to undergo organogenesis during which begins the initial formation of the organs.

The embryo is generated by activation of a mature oocyte usually by sperm and so in order for the process of embryogenesis to be embarked upon an oocyte has to reach a state in which it is able to be fertilised.

The process of oogenesis involves the primordial germ cell-derived oogonia in the gonad of the vertebrate undergoing a series of changes and divisions to produce variable numbers of the mature egg (ovum).

Following the multiplication of oogonia by mitosis within the animal ovary differentiation ensues forming primary oocytes. The oocyte then enters into the first of the two divisions of meiosis it experiences in order

to become a haploid gamete. Homologous chromosomes pair and crossing over occurs. This primary oocyte can then remain arrested in this stage for a variable period depending on the species. The oocytes can accumulate material necessary for growth in early embryogenesis during this arrested period. In *Xenopus*, the oocyte progresses through a series of changes which have been characterised and defined as 6 stages known as Dumont stages. (table 1)

Stage	I	II	III	IV	V	VI
Size (µm)	50-300	300-450	450-600	600-1000	1000-1200	1200-1300
Appearance	Transparent	White, opaque	Light brown	Pigmented animal pole	Clear boundary between hemispheres	Equatorial belt visible

Table 1: Dumont-based classification of growing *Xenopus* oocytes (information from Hausen and Riebesell (1991)). Original classification in Dumont (1972).

On hormonal induction, the primary oocyte resumes the progress through meiosis and is said to be undergoing maturation. After separation of homologous chromosomes into daughter nuclei, the oocyte divides distributing one nucleus to the secondary oocyte and the other to a much smaller polar body destined for degradation. This minute structure can be observed to be extruded from the oocyte outer membrane by exocytosis (Hausen and Riebesell, 1991). The sequence of changes during oogenesis and maturation are represented in figure 1.

The separation of chromosomes (in the form of chromatid pairs) during the second meiotic division may not follow directly as a result of a second arrest whereby the oocyte halts at metaphase II. The release from this arrest does not occur until fertilisation when meiosis is completed.

Another polar body is produced in an asymmetric division containing the second set of chromatids. The mature egg, which will then become a zygote, is the principal end-product of the maturation and fertilisation process.

Xenopus laevis, the South African clawed frog, is characteristic of the above described general account of vertebrate oocyte maturation in many respects and has several advantages which have led to its widespread use and it becoming an established experimental model system. The *Xenopus laevis* oocyte is of a massive size relative to human oocytes for example and is quite resistant to manipulation making it well-suited to biochemical and molecular studies of oocyte maturation.

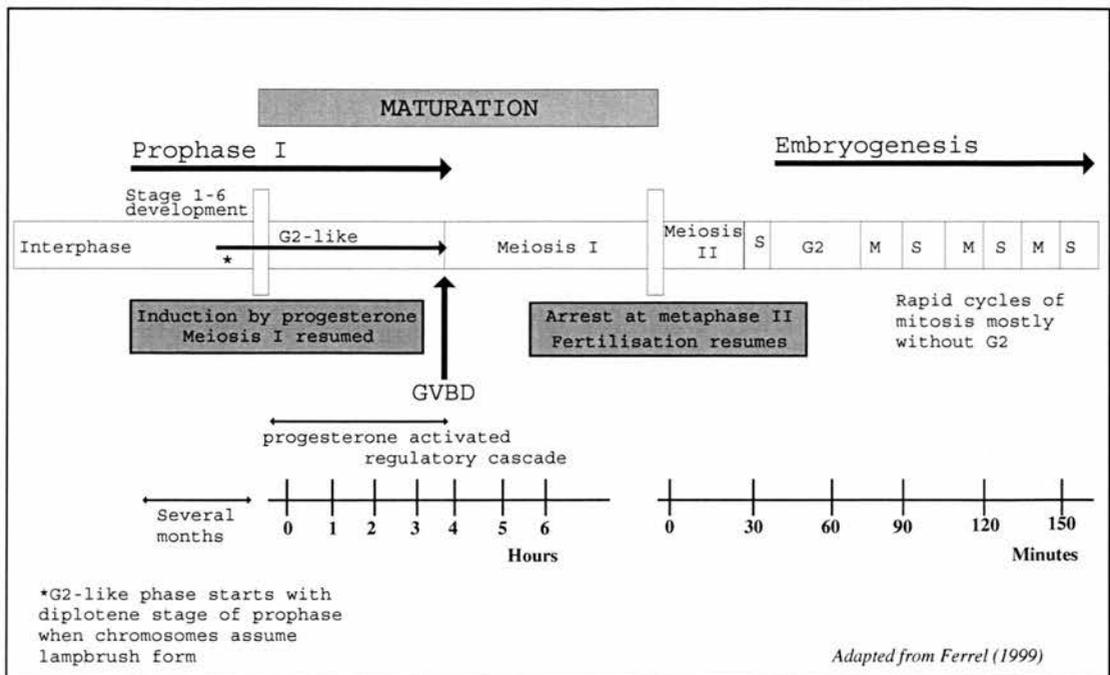


Figure 1: Sequence of changes occurring during *Xenopus* oogenesis and maturation.

The process of oocyte maturation in *Xenopus* is induced by the hormone progesterone, produced after an appropriate external cue has been received by the animal. This signal is transduced into a series of partially delineated biochemical changes which ultimately bring about activation of a functional maturation-promoting factor (MPF), the protein complex that triggers continuation through the meiotic nuclear division (Haccard *et al.*, 1995). This role is widespread through animal cells and MPF has been described as the the 'master cell-cycle switch' (Gebauer and Richter, 1997). This series of maturation-associated biochemical changes warrants further explanation to gain an insight into the key mediators and regulators of the process particularly the aforementioned MPF and the protein Mos. Their

role seems to be central to maturation and their regulation is critical to the key events of germinal vesicle breakdown (GVBD) during the 1st nuclear (meiotic) division and the second meiotic arrest (Nishizwa *et al.*, 1992).

Indeed it is the nuclear dissolution of GVBD that is used as a visible indicator of maturation in *Xenopus* oocytes where pigment granules beneath the cortex of the oocyte's animal pole become rearranged, resulting in the appearance of a white spot.

The process of maturation has been summarised as the activation of a progesterone receptor on the plasma membrane of the oocyte which leads to the activation of the MAPK (mitogen-activated protein kinase) cascade, of which Mos is a member, in turn leading to activation of Cdc2, a component of MPF which releases the oocyte from the first meiotic arrest leading to GVBD (reviewed in Ferrell, 1999).

The binding of progesterone to the *Xenopus* oocyte progesterone plasma membrane receptor induces several biochemical changes. One significant action is the inhibition of the enzyme adenylate cyclase - which reduces the concentration of the intracellular signalling molecule cyclic AMP (Maller *et al.*, 1979). This in turn leads to a decrease in protein kinase A activity whose reduction experimentally can induce maturation of *Xenopus* oocytes in the absence of progesterone (Maller and Krebs, 1977). Other

effectors of maturation downstream of progesterone but upstream of the MAPK cascade have been implicated such as the kinase Eg2.

Andresson and Ruderman (1998) screened for *in-vitro* translated, injected proteins in oocytes which become modified, say by phosphorylation, soon after progesterone stimulation. These proteins were identified by a shift in their mobility during SDS-PAGE relative to the unmodified proteins.

Although shifting induced by post-translational modification is not universally observed with proteins, Eg2 was reported as one of the shifted proteins whose sequence was already known and oocyte injection with the corresponding mRNA was reported to accelerate the appearance of the Mos protein in the oocyte. This indicated Eg2 as a member of the pathway linking activation of the surface progesterone receptor with the translational recruitment of the stored mRNA for Mos. This early role was supported by the findings of Mendez *et al.* (2000) who reported that Eg2 was necessary for the phosphorylation of CPEB, a protein necessary for the polyadenylation of several stored mRNAs including *mos* (polyadenylation being another major mechanism for translational control of stored mRNAs).

The importance of Eg2 however was later brought into question by the work of Frank-Vaillant *et al.* (2000) with results that appear to refute much

of the findings of Andresson and Ruderman and Mendez *et al.* which describe the accumulation but not the activation of Eg2 to be a consequence of progesterone stimulation before maturation. Frank-Vaillant *et al.* further report that the activation of Eg2 occurs subsequent, that is downstream, of MPF activation. The function of Eg2 was therefore speculated to be related to microtubule-associated events during meiosis given that it is known to function in mitosis, associating with tubulin at the poles of the mitotic spindle in somatic cells.

This apparent conflict of results makes explaining the reported effects of Eg2 difficult particularly the apparent requirement of *mos* mRNA polyadenylation for the kinase (Mendez *et al.*, 2000). Perhaps, as stated by Andresson and Ruderman (1998), the distribution of Eg2 at the oocyte cortex — where the progesterone receptor is thought to reside — may give some understanding of why Eg2 potentiates the maturation process. This potentiation might be a spurious effect of experimentally overexpressing the kinase given the multiple roles acknowledged by Andresson and Ruderman in which a kinase of a single cell-type may partake. The kinase, perhaps significantly also belongs to the aurora kinase family whose other members include Aurora in *Drosophila* and Ipl1 from budding yeast. These two proteins are known to be involved in preparing for mitosis for

example in spindle assembly and also chromosome condensation during the final stages of interphase in higher eukaryotes (Pines and Rieder, 2001). This mitotic role suggests Eg2 is important in maturation but perhaps adds less weight to the notion of Eg2 as an early mediator of maturation.

Whatever the precise nature of events that transduce the external binding of progesterone to the oocyte membrane to the MAPK cascade, the translational activation of *mos* mRNA is a central event in the maturation process (Charlesworth *et al.*, 2002), although whether it is critical for maturation to occur in *Xenopus* has been disputed recently (Dupre *et al.*, 2002). It is understood to be the MAPK that contributes to the increase in kinase activity that can be observed before GVBD and thereby distinguishes these biochemical changes from the early events following progesterone binding.

Mos is a MAP kinase kinase kinase which refers to its position in the MAPK cascade transferring phosphate to, and thus activating, the next kinase (MEK1, a MAP kinase kinase). Mos can function as both an inducer of maturation and as a so-called cytostatic factor depending on the organism. For instance there are differences in the function of Mos between *Xenopus* and mice. In *Xenopus* Mos activates the MAPK cascade

and in doing so leads to MPF activation and GVBD. Mos has further roles however in *Xenopus* (reviewed in Gebauer and Richter, 1997). It suppresses DNA replication between meiosis I (1st division) and meiosis II (2nd division) in the maturing oocyte by re-activating MPF which declines in activity after metaphase I. In interphase of somatic cells dividing mitotically, MPF activity remains low allowing DNA replication to take place. Also Mos has cytostatic activity as it is required along with other components to maintain the cell-cycle arrest at metaphase II (Sagata, 1997). The changes in Mos activity during oocyte meiosis and the mitotic divisions of early embryogenesis are represented in figure 2.

In certain other species such as the mouse and goldfish, MPF activation and maturation does not appear to require the activity of Mos and the MAPK cascade but Mos activity is thought to be necessary for metaphase II arrest as in *Xenopus* (Verlhac *et al.*, 1994; Kajiura-Kobayashi *et al.*, 2000). Its downregulation at the end of metaphase II (*i.e.* on egg fertilisation — 'activation') relies upon both degradation by the ubiquitin-tagging dependent protein degradation pathway (Nishizawa *et al.*, 1992) and cessation of translation by deadenylation (Ueno and Sagata, 2002). Recent work by Dupre *et al.* (2002) has shed more light on the significance of Mos in the various roles it is associated with in *Xenopus* and the

discrepancies in these roles with certain other studied species. By injecting morpholino antisense oligonucleotides (short nucleic acid molecules complementary to the translation start region of a particular mRNA which block access of the translation machinery to the mRNA) Dupre *et al.* were able to eliminate all detectable Mos protein expression yet still observe GVBD and MPF activation (*i.e.* physical and molecular proof of maturation) following exposure to progesterone albeit delayed compared to non-injected oocytes. Additionally the results indicated that MPF activation was possible in the absence of Rsk activation (previously thought a possible link between the MAP kinase cascade and MPF activation) and that an alternative pathway additional to the MAPK cascade contributed to Rsk activation. Emerging from the work of Dupre *et al.* was a more likely role for Mos in the establishment and maintenance of cell-cycle arrest at metaphase II rather than an essential factor for maturation / completion of meiosis I. In oocytes with Mos ablated the normal reactivation of MPF required for suppression of DNA replication, as occurs between mitotic divisions, did not occur and consequently oocytes started to synthesise DNA. Therefore from this work it seems that Mos is essential in arresting the progression of the oocyte cell cycle at metaphase II until fertilisation despite it contributing to the rate at which

maturation is instigated following progesterone exposure. In other words Mos may be preventing parthenogenesis (activation of the oocyte / zygote development without fertilisation) which would appear to be the common role across species such as *Xenopus* and mouse. Rsk protein thus may similarly be an essential component of the reactivation of Cdc2 after meiosis I. Apparently therefore, the identity of the protein or proteins essential for initial Cdc2 activation and subsequent maturation following progesterone stimulation remain to be identified.

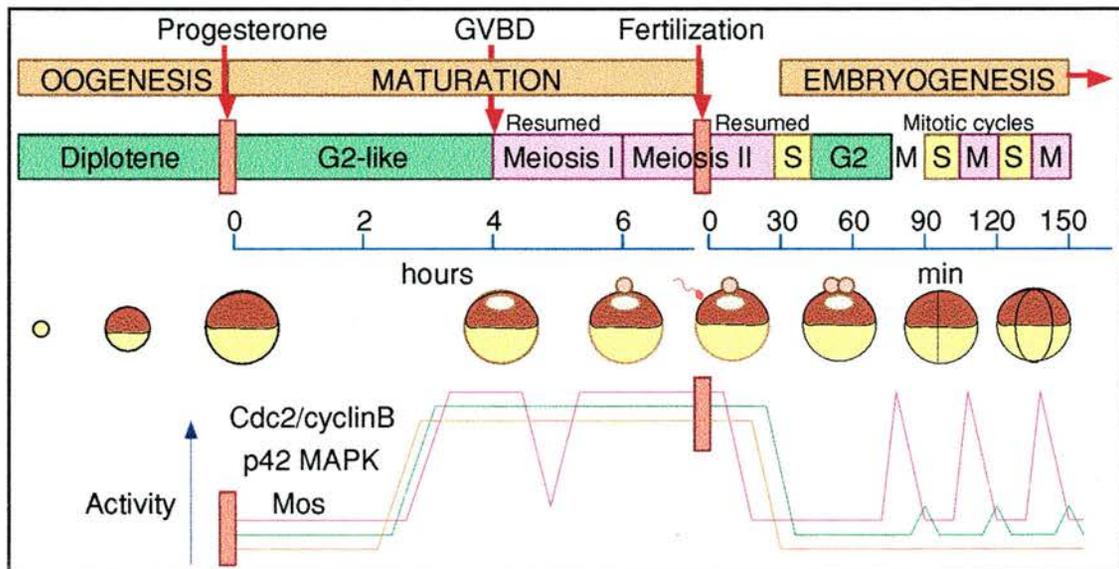


Figure 2 :Changes in Mos and MPF activity during oogenesis meiosis and mitosis of early embryogenesis.

The relative levels of activity of two of the components of the MAP kinase cascade (Mos and p42 MAPK) and MPF (Cdc2/cyclinB) are shown alongside the stages of *Xenopus* oocyte maturation, fertilisation and early embryogenesis.

Note the arrest of the oocyte in the diplotene stage of meiotic prophase (which resembles a cell cycle gap phase), the resumption of the cell cycle by progesterone treatment, the second arrest at metaphase II, the reactivation of MPF towards the end of meiosis one and the cycling of its activity during the mitotic divisions of embryogenesis. Also note the inactivation of Mos outside of meiosis.

The developing oocyte, the mature egg, zygote and cleaving zygote are shown together with emerging polar bodies at the end of meiosis I and meiosis II.

On activation by Mos kinase activity the MEK1 protein activates the MAP kinase p42/ERK2 or p44/ERK1 depending on the vertebrate organism, the former being present in *Xenopus* oocytes (Posada *et al.*, 1991). The MAP kinase cascade then activates MPF indirectly through p42/p44's kinase action on intermediate factors (reviewed in Ferrell, 1999). One such factor may be one of the Rsk proteins which are downregulators of a major Cdc2-inactivating factor, Myt, and in *Xenopus* at least have been experimentally associated with MAPK activation (Palmer *et al.*, 1998).

An active MPF then brings about the changes characteristic of maturation notably chromosome condensation, spindle formation and GVDB by phosphorylating molecules such as histones and nuclear lamins.

There exists a positive feedback loop between MPF and Mos whereby the activation of p42/p44 MAPK and MPF increase the levels of Mos protein by stimulating *mos* mRNA translation or stabilising the Mos protein. This feedback loop may be essential to raise the level of Mos protein sufficiently to commit the oocyte to completing maturation (Chen and Cooper, 1997). The translational activation of *mos* mRNA appears to be mediated through different mechanisms by MAPK and MPF (Charlesworth *et al.*, 2002) although the end result of both mechanisms is polyadenylation of the *mos* transcript thereby stimulating its translation.

The work of Charlesworth *et al.* identified a novel regulatory element in the 3' UTR (untranslated region) of *mos* mRNA which they termed the *polyadenylation response element* (PRE) and which responds to MAPK activity in *Xenopus* oocytes early after progesterone stimulation. This was found to occur independently of and prior to Cdc2 (a component of MPF) activation of *mos* translation, a mechanism dependent on the cytoplasmic polyadenylation element also present in the *mos* mRNA 3' UTR. This pathway also involves the kinase Eg2 which phosphorylates the CPE-binding protein (CPEB) enabling it to mediate the polyadenylation of the transcript. However, CPEB-mediated translation is necessary to drive the oocyte to maturation (Charlesworth *et al.*, 2002).

The positive feedback mechanism also involves p42 MAPK in *Xenopus* reducing the degradation of Mos protein by chemically stabilizing it through phosphorylation (Nishizawa *et al.*, 1992) and a similar mechanism through direct Cdc2 phosphorylation of Mos (Castro *et al.*, 2001).

It is not yet clear if MAPK causes the initial accumulation of Mos to establish the MAPK loop or if it is a result of a further pathway or gradual low-level translation. If the MAPK activates the initial Mos translation then the pathway which activates MAPK initially remains to be characterised.

Export of mRNA from the nucleus

The intense transcription that occurs during vertebrate oocyte maturation must ultimately result in the formation of cytoplasmic mRNP particles.

This therefore necessitates the existence of mechanisms to assemble the mRNP complexes, to export the complexes from the nucleus and to direct them into the cytoplasm at specific locations if required. As may be expected the relatively lower degree of translation at this time means that certain proteins aren't available in large quantity to enter organelle-mediated localising and accumulation for subsequent embryogenesis.

More importantly though perhaps the mRNA molecules can be stabilised more easily in the form of mRNP particles than proteins.

The export of mRNA is particularly well-studied in oocytes of *Xenopus* and *Drosophila*. However, the study of the mRNA localisation process, which is believed to be closely linked to the events occurring before and leading up to nuclear export, has revealed much about the export machinery of diverse eukaryotic organisms such as yeast, mammals and protozoa and within the somatic cells of metazoans (reviewed in Jansen, 2001). RNA export mechanisms are also exploited by viruses such as the human immunodeficiency virus (HIV) making the topic an area of keen research activity.

The function of mRNA localisation in oocytes is most significantly to deposit the genetic information required for producing determinants of cell fate in order to form concentration gradients within the oocyte that are necessary to establish the embryonic body plan. Numerous examples are known mainly from *Drosophila* and *Xenopus* such as *bicoid* mRNA which localises to the anterior of the *Drosophila* oocyte to form a concentration gradient of bicoid protein which acts as a morphogen during embryogenesis. The *Vg1* mRNA localises to the vegetal pole of *Xenopus* oocytes. The situation in *Drosophila* is complicated by the apparent necessity for certain mRNAs such as *bicoid* to form mRNP particles in the nurse cells of the egg chamber before they will localise in the oocyte on subsequent export from the nurse cells (reviewed in Arn and Macdonald, 2001). However the principle remains of proteins binding to the mRNA before export acting as tags to allow localisation machinery in the cytoplasm of the oocyte to move or immobilise the RNA (although *Vg1* of *Xenopus* relies upon a binding protein, vera, for microtubule-based localisation then short RNAs for immobilisation; Kloc and Etkin, 1994). In order to describe models for RNA export it is necessary to introduce the nature of proteins to which many RNAs are bound. It is important to note though that different types of RNA (small nuclear RNA, messenger,

transfer and ribosomal) operate through the use of different export factors or almost entirely distinct mechanisms (reviewed in Ullman (2002) and Cullen (2003)). The transport of proteins into and out of the nucleus is mediated by amino-acid sequences residing within the protein which interact with one or more factors to enable passage through the nuclear pore (protein-) complex (NPC), a complex of 30-50 proteins known as nucleoporins with extensions and fibrils into the cytoplasm and nucleoplasm acting as docking sites for cargoes undergoing nucleocytoplasmic transport. Until recently most information on this protein-trafficking has been available for a well-defined set of nuclear-localisation signals (NLSs) and nuclear export signals (NESs). The classical NLSs are characterised by a rich content of basic amino acids whereas the consensus NES is a stretch of leucine residues. An example is given in figure 3 of a key mRNP protein in *Xenopus* and the conservation of the NES signal with HIV Rev protein (therein exploiting cellular machinery). The factors and events involved in the transport of classical signal-containing proteins has been widely studied. Import of NLS-containing proteins is mediated by the cytoplasmic protein importin- α which forms a complex with the protein and another factor, importin- β . The complex is then able to interact with the NPC enabling localisation to

the nucleus where it may remain permanently or be exported. Numerous studies have also revealed the identity of the export receptor, CRM1 mediating translocation of the NES-containing proteins to the cytoplasm. Indeed nucleocytoplasmic transport of proteins and RNA have various similarities and it is thought that most of this kind of movement in the cell is mediated by the class of transport receptors known as karyopherins of which CRM1 is a member. Karyopherins all have the ability to bind to nucleoporins and depend upon a gradient of activity of the GTPase Ran, an enzyme with which they also associate. The export of NES-bearing proteins using CRM1 however appears to be more relevant to the export of RNAs such as snRNAs and not to the export of mRNAs (Cole, 2000). Certain mRNAs however are exported from the nucleus using CRM1. For example certain viruses exploit the CRM1 protein such as the HIV Rev protein mentioned above whose NES interacts with CRM1 and enables incompletely spliced mRNAs to exit the nucleus. Only in certain metazoan tissues is there thought to be cellular mRNA nuclear export dependent on CRM1. A protein related to the major metazoan mRNA nuclear export factor Tap called NFX3 bears a leucine-rich NES and can associate with CRM1 and poly(A)⁺ mRNA in certain tissues (Yang *et al.*, 2001).

N	G	T	G	K	T	G	A	Y	L	I	P	L	L	E	R	L	D	L	K	160
									Q	L	P	P	L	E	R	L	T	L	(HIV)	

Figure 3: The leucine-rich putative NES in Xp54 (top) and homology with the HIV REV protein.

An understanding of a distinct mechanism for the export of mRNA from the nucleus has begun to emerge involving numerous dedicated export factors many of which are deposited onto the mRNA molecule prior to movement through the NPC. The soluble factors involved in the process of splicing are closely linked to mRNA export and also the subsequent stability during translation in the cytoplasm. This functional association is discussed further below. However it has recently been indicated that more than only splicing might be necessary to distinguish an RNA molecule as messenger RNA to send it through the mRNA export pathway. For example, an unstructured, non-sequence specific stretch of RNA of around 300 nucleotides was found to allow a hybrid snRNA to be transported through the NPC by the set of factors normally specific to the vast majority of - spliced intron-containing and non-intron containing - mRNAs (Ohno *et al.*, 2002). This element although loosely defined would presumably only be characteristic of the messenger form of RNA although the mechanism for its recognition remains to be characterised.

One key protein involved in passage of mRNA through the NPC is dbp5p

found in yeast and mammalian cells and is thought to release the mRNA (in the form of mRNP) from the NPC to the cytoplasm (Snay-Hodge *et al.*, 1998), thereby allowing shuttling export factors to be released from the mRNP and return to the nucleus. Dbp5p is a member of the DEAD-box protein family which is a family of ATP-dependent RNA helicases bearing the DEAD amino-acid motif in their sequence. Such helicases function throughout RNA metabolism in cells for instance splicing, export and translation. In yeast, and presumably in close similarity to metazoan cells, numerous other factors are involved in conveying the mRNA to the NPC in a chain of binding and release events (reviewed in Linder and Stutz, 2001). These factors include yra1p, mex67p, mtr2p and sub2p (figure 4 and table 2). However the key proteins are understood to be mex67p/TAP and its cofactor mtr2p/nxt around which the proteins involved in other mRNA-associated nuclear mechanisms centre. These proteins eventually recruit mex67p and mtr2p to export the bulk of mRNA (Cullen, 2003).

Metazoan name	Yeast name
TAP	Mex67p
Nxt	Mtr2p (p15)
Aly/REF	Yra1p
UAP56	Sub2p
Table 2: Names of mRNA nuclear export factors in metazoans and yeast	

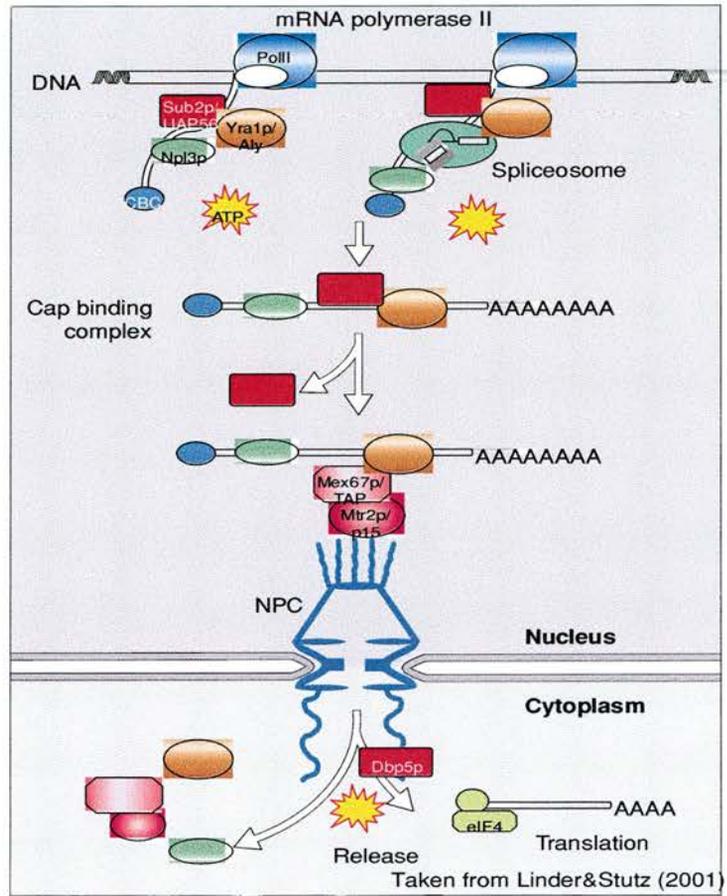


Figure 4: The series of soluble factors operating to deliver an mRNA molecule through the NPC to the cytoplasm for translation (see text for details).

At the time of export factor activity (which appears to start during transcription) the mRNA is already in the form of mRNP with various proteins bound to its surface such as cap binding protein and splicing factors.

Yra1p (Aly in mammals) is an RNA export factor-binding protein and recruits the shuttling receptor mex67p to the mRNP. Aly, itself is recruited by UAP56 (the mammalian homologue of sub2p) and binds to the mRNA during splicing of intron-containing genes as UAP56 is a component of the spliceosome protein complex (Kistler and Guthrie, 2001) but is able to interact directly with Aly (Luo *et al.*, 2001). The subsequent recruitment of mex67p displaces sub2p. Sub2p/UAP56 appears to have a dual role in being involved in both RNA export and RNA splicing (Libri *et al.* 2001). However, the two roles may well have a close link functionally, at least in intron-containing genes (although sub2p is apparently deposited on intron-containing and intron-less genes). Linder and Stutz (2001) postulate that sub2p may be involved in the release of mRNP from the site of transcription ensuring only properly spliced mRNAs are allowed to proceed to export.

Before export mex67p forms a heterodimer complex with mtr2p enabling the mRNP to bind to the NPC with dbp5p using ATP-dependent helicase

activity to release the mRNP into the cytoplasm for translation. Again it is important to state that the generalised mRNA nuclear export process described above is different to the route of NES-motif containing proteins using the CRM1 receptor protein which were initially proposed to carry mRNA to the cytoplasm (reviewed in Cole, 2000).

Other factors are known to be important in mRNA export and more are likely to be discovered as the above described mRNA export system begins to be fully understood. One factor which apparently has a key role however is Y14 from yeast which can be found on spliced and intron-less mRNA (Ohno *et al.*, 2002) which may act as a general mRNA adaptor protein. It is also part of the exon junction complex which is deposited on spliced mRNA regions of intron-containing genes (Le Hir *et al.*, 2000) and has a role in localisation of the *Drosophila* mRNA *oskar*, whose protein is a major determinant of embryonic development in the organism (Hachet and Ephrussi, 2001). This suggests multiple roles for this mRNA-binding protein in a similar way that UAP56/sub2p is involved in splicing of intron-containing genes and export of mRNA both intron-containing and intron-less genes.

Other processes which are thought to lead to the recruitment of mex67p/mtr2p include transcription and mRNA 3' end formation. It is thought that

these processes together with splicing and any mRNA identity elements that might exist are united by sub2p/UAP56 as opposed to various other factors discussed above. Sub2p is the common factor that is recruited in apparently all cases amongst various metazoan organisms and yeast (Cullen, 2003).

During transcription export factors may be recruited to the mRNA such as yra1p and sub2p associating with the transcription elongation complex THO in yeast (Strasser and Hurt, 2002). However there must exist a means to ensure that precursor mRNAs are spliced and are not allowed to be recruited by mRNA export factors. Various data exists indicating an association between the formation of a mRNA poly(A) 3' end and export although the exact nature of this association is unclear. Rather, the consequences with regards to mRNA export of experimentally altered mRNA 3' ends and the effects on 3' ends from defective export factors appear to vary between experiments or species (Cullen, 2003).

Role of splicing components in the stability of exported mRNA

As stated above, most intron-less genes as well as spliced mRNAs appear to share an export route mediated by common factors some of which are involved in splicing. These factors however operating on spliced genes

influence the translation and stability occurring downstream of export especially by recording the accuracy of splicing which is used by *mRNA surveillance* mechanisms in the cytoplasm. Although other proteins may bind to the mRNA in the nucleus and accompany the mRNA into the cytoplasm to influence stability subsequently it is the splicing components that are thought to be used by surveillance mechanisms to detect premature translation termination codons (nonsense codons).

The reason for the existence of such a mechanism is that nonsense codons which appear in mRNA as a result of a mistake in transcription or aberrant splicing can potentially generate peptides that have are toxic and therefore deleterious to the cell so it is to the cell's advantage to detect the mRNA precursors of such peptides. The mechanism in eukaryotes is known as nonsense-mediated mRNA decay (NMMD) and on detection of a nonsense codon the normal mRNA decay process is bypassed to accelerate the decay of the relevant mRNA. NMMD has been well-studied in yeast and to slightly less an extent in mammals.

There is a lower proportion of intron-containing genes in yeast than in mammals and studies in mammals have revealed the links between splicing events and factors and NMMD. However there are numerous homologues in the detection and decay process of NMMD in both groups

of organisms and also in the worm *Caenorhabditis elegans* (Serin *et al.*, 2001) indicating that NMMD is a well-conserved process.

In mammals following the removal of introns by the spliceosome, exon junction complexes are deposited marking the regions from where the introns were excised. If such a marker is detected by surveillance factors following translation termination at a position more than approximately 50 nucleotides downstream of the termination codon then the decay process is instigated with concomitant rapid degradation of the mRNA (reviewed in Mitchell and Tollervey, 2001). This relies on most mRNAs not possessing introns within their 3'UTR. The set of 3 proteins, Upf1p, Upf2p and Upf3p are known to be key components of yeast mRNA surveillance with homologues in humans termed hUpf proteins. The distribution of these proteins is such they overlap in the peri-nuclear region which is where NMMD seems to occur in higher eukaryotes (Ross, 2001). hUpf3p is exported with the spliced mRNA and then interacts with hUpf1p and hUpf2p (hUpf1p being recruited to the mRNA at translation termination). The binding of hUpf3p during splicing in the nucleus functionally links the process with mRNA surveillance and thus the stability of mRNA within the cytoplasm.

It is during the first run through translation that many of the proteins that

bind to the mRNA in the nucleus such as those of the exon junction complexes are removed by the passage of the ribosome. As shown in figure 5 following termination of translation the translation release factors recruit Upf1p as part of a surveillance complex which is then thought to move along the 3'UTR scanning for the presence of Upf3p and exon junction markers. The surveillance complex then is thought to trigger decapping and resulting rapid degradation by interaction with initiation factors and Upf2p which involves looping round of the mRNA molecule as illustrated. In the absence of aberrant splicing markers the mRNA is circularised by the action of initiation factor eIF4E which replaces the cap-binding complex at the 5' end of the mRNA and enables binding of the 5' end to the polyadenylate binding protein attached to the polyadenylated 3' end of the molecule. This structure promotes further rounds of translation. Differences exist between mammalian cells and *Xenopus* oocytes in the composition of the exon junction complexes possibly because of the reported lack of NMMD in *Xenopus* at this early developmental stage of the organism (Lejeune *et al.*, 2002). The picture is further confused by reports from Lejeune *et al.* that some aberrant mRNAs are found in the nucleus (as opposed to the cytoplasm) apparently at a stage in the NMMD process that would indicate that translation has occurred and the passage of

the ribosome has removed the exon junction complexes. Pre-mRNA (intron-containing) is thought to be normally bound by the mainly nuclear cap-binding protein CBP80 and following splicing an initial round of translation determines whether there will be NMMD triggered depending on the presence of premature termination codons. CBP80 is then replaced by the mainly cytoplasmic eIF4E after removal of the exon junction complexes. However eIF4E-bound spliced mRNA can be found in the nucleus of mammalian cells suggesting the NMMD process might be more complex than at first thought. The cap protein replacement however might occur as the mRNA is being exported into the cytoplasm and so have access to ribosomes to carry out a first translation round and remove exon junction complexes.

Figure 5 summarises the processes involved in NMMD-associated mRNA remodelling including the key protein components of the exon junction complex in mammalian cells and the differences in *Xenopus* oocytes.

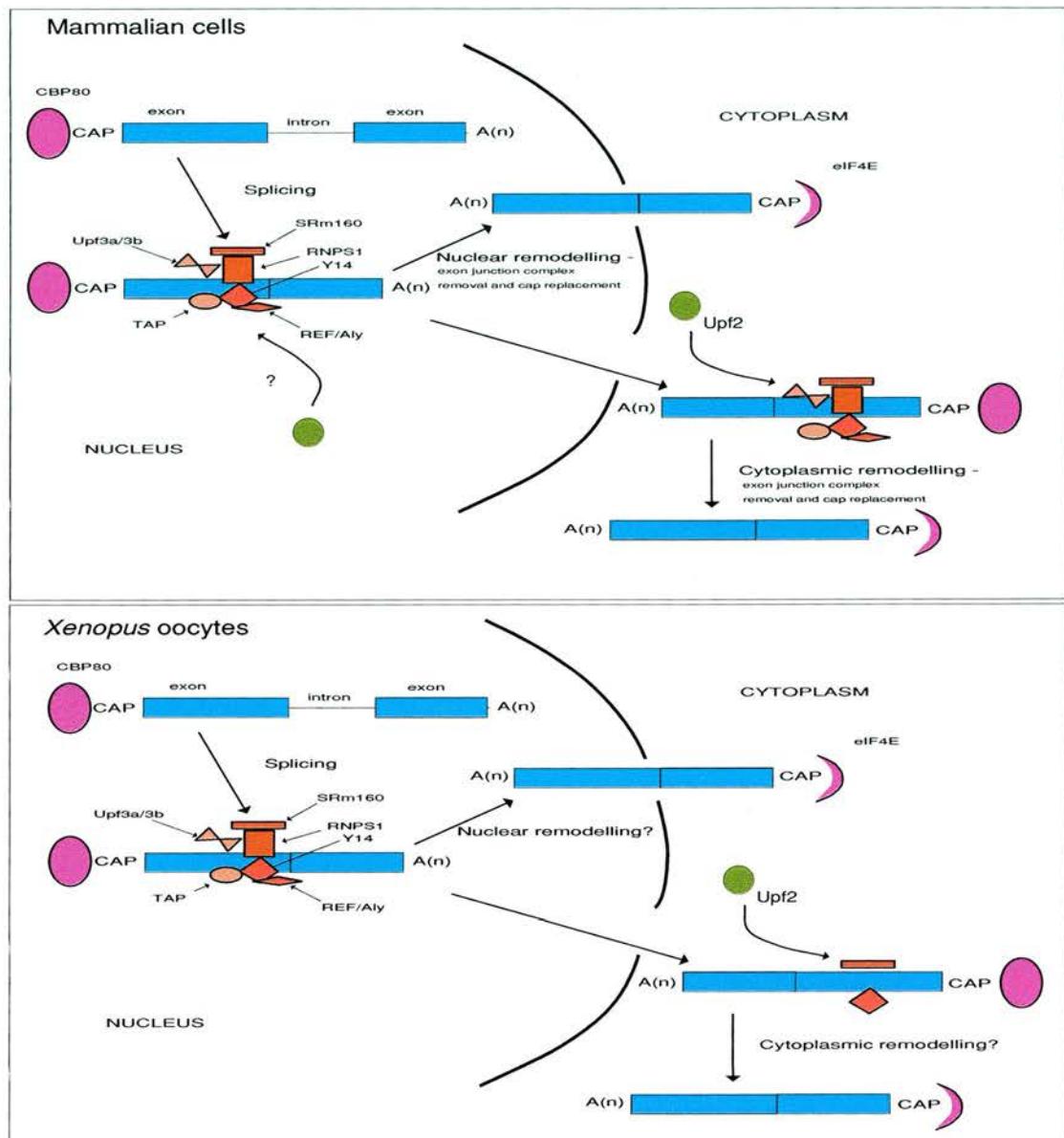


Figure 5: NMMD-associated mRNA remodelling in mammalian cells and *Xenopus* oocytes.

The sequence of processes and the differences between the organisms are based on the model and diagram presented in Lejeune *et al.* (2002). In both organisms the vast majority of pre-mRNAs are bound at the cap by CBP80. Splicing then occurs and exon junction complexes (components represented by dark orange shapes) bind 20-24 nucleotides upstream of exon-exon junctions. Spliced mRNAs then undergo remodelling in which CBP80 is replaced by eIF4E at the same time or after the EJC is removed. In the case of nuclear-modelling this is assumed to take place as the 5' end of the mRNA exits the nucleus when the mRNA has access to ribosomes. The NMMD-associated factor Upf2 has so far only been detected on mRNAs leaving the nucleus and in the cytoplasm or freely in the cytoplasm and is mainly perinuclear. The NMMD factor Upf3a/b is recruited by the exon junction complex along with the mRNA export factor TAP which interacts with Aly to export the mRNA. However in *Xenopus*, TAP and the exon junction components RNPS1 and Aly are not detected on exported mRNA nor is Upf3a/b whilst Upf2 is found only on cytoplasmic mRNAs. The lack of detectable NMMD in *Xenopus* oocytes reported by Lejeune *et al.* might underlie these differences.

The above is a generalised model and would be expected to apply more to mammals as most yeast genes in fact lack introns. Also there are certain mammalian genes without introns but there exist additional pathways which can identify premature translation termination codons or incorrectly spliced mRNAs. For example there exist *downstream sequence elements* (DSEs) in yeast which are thought to trigger nonsense-mediated decay. The protein Hrp1p for instance binds to a DSE in the yeast PGK1 gene and interacts with Upf1p (Gonzalez *et al.*, 2000). Hrp1p in fact appears to be necessary for NMMD in yeast and Wilusz *et al.* (2001) propose that it acts as a 'tag' identifying potential premature termination codons. When bound to DSEs within coding regions the interaction would be ended by the ongoing movement of the ribosome but when hrp1 is bound to a DSE downstream of a premature stop signal there would be no such disruption and the protein would be able to proceed with instigating NMMD. So-called fail-safe sequences may be a higher eukaryotic equivalent to DSEs in yeast although Mitchell and Tollervy (2001) suggest that these sequences may be *exonic-splicing enhancers* located near to the splicing site, presumably to accommodate for the possibility of the splicing and marking process not working in some way or the mRNA surveillance process failing to recognise the spliced region.

The true complexity of the 'interpretation' of mRNA-bound nuclear proteins by cytoplasmic processes such as translation is still beginning to emerge. The induction of decapping by the Upfp complex for example may not be subject precisely to the same mechanism that has previously been studied wherein the major yeast decapping enzyme Dcp1p outcompetes initiation factor eIF4E for the binding of eIF4G bound to the cap (Schwartz and Parker, 2000). The rapid decapping following mRNA surveillance is not inhibited by mutations in a complex array of proteins which are necessary for this process, including the lsm proteins (Bouveret *et al.*, 2000). One possible model proposed by Mendell *et al.* (2000) for NMMD-induced decapping involves Upf2p displacing eIF4G and allowing easier access to the 5' cap for a decapping enzyme. Exactly how the surveillance complex and thus in turn how the Upf3p splicing factor and homologues are linked to (*i.e.* how they induce) the decapping activity of Dcp1p, described as the 'eukaryotic decapping protein' (Vilela *et al.*, 2000), or even other decapping activities remains to be fully described.

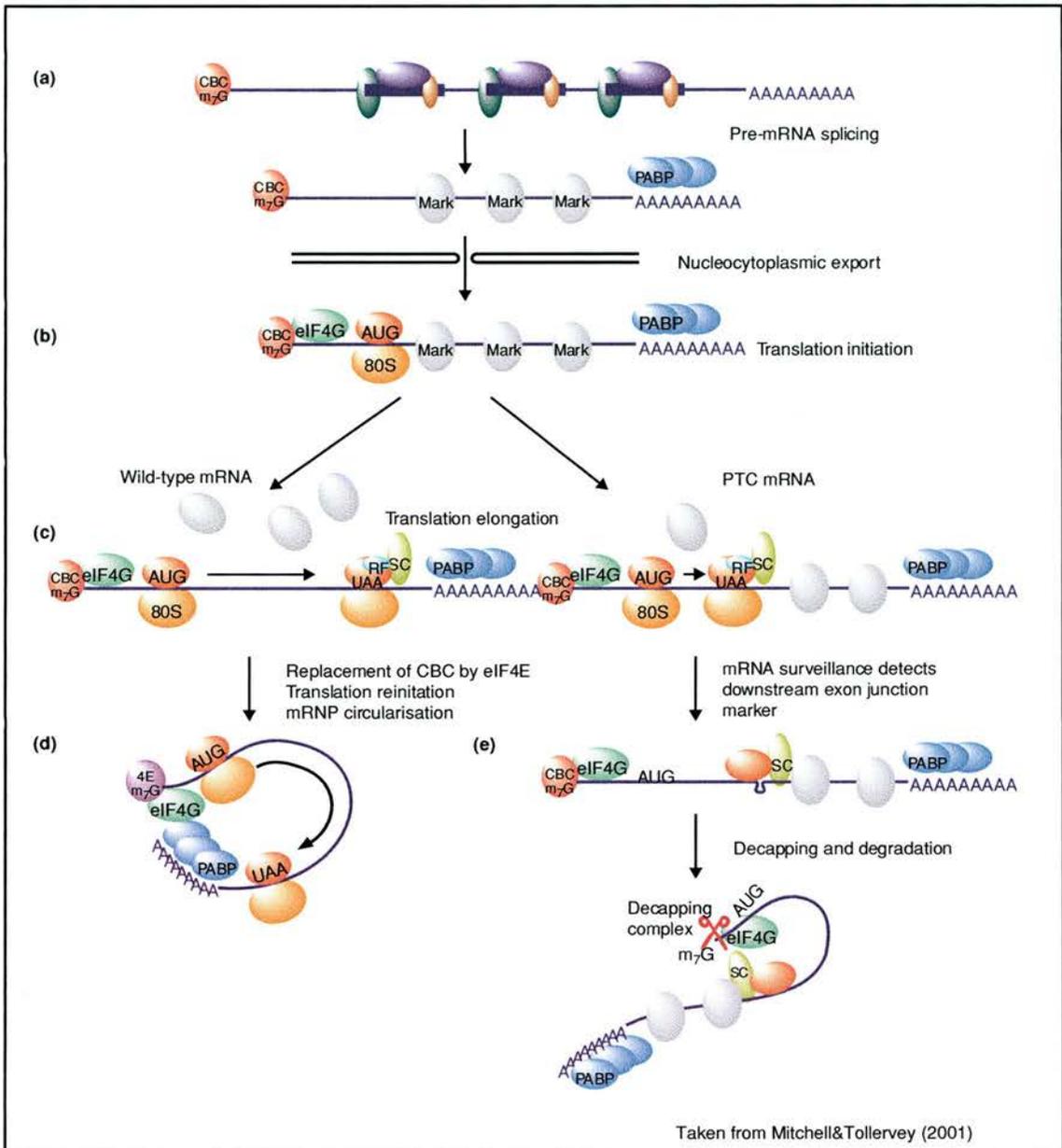


Figure 6: The mRNA surveillance process in a typical mammalian intron-containing gene.

(a) Nuclear splicing of pre-mRNA by spliceosomes and assembly of exon-junction marker complexes on spliced sites. (b) Assembly of translation initiation complex including cap-binding complex (CBC) and translation of mRNA by ribosome. (c) Surveillance complex recruited to mRNA by translation release factors (RF) which moves through 3' UTR. (d) If no inappropriate marker proteins encountered mRNA adopts 'mature' mRNP structure. (e) If incorrect marker proteins are encountered the surveillance complex triggers decapping and rapid degradation of the mRNA.

Stability of mRNA in the cytoplasm

In addition to mechanisms that function to detect and remove the effects of mistakes that occur during transcription or splicing by affecting mRNA degradation, the eukaryotic cell possesses the ability to adjust specific or general mRNA decay rates (mRNA turnover) in response to external stimuli such as hormones, cytokines or infection. This constitutes an important means of controlling the expression of the gene by limiting the total amount of a protein produced from a gene and determining the timing at which the translation from a particular transcript ceases completely.

The decay rate of mRNA is commonly described as its half-life, the length of time for particular RNAs to decay by half in quantity. This varies widely and in yeast for example can occur within a fraction of the cell-cycle or over several cell cycles (Herrick *et al.*, 1990). The regulation and functioning of decay pathways requires messenger RNA ribonucleases operating in distinct decay processes, regulatory factors and sequence elements within mRNA molecules that are able to respond to regulatory factors.

There are certain distinguishable classes of mRNA decay common to widely separated organisms (reviewed in Ross, 2001). Common to both metazoans, yeast and bacteria are 3' to 5' exonucleases but this pathway is

thought to be more important in bacteria. Also important to prokaryotic mRNA decay is endonucleolytic cleavage but this is also a proven metazoan decay pathway for certain mRNAs. For example α -globulin mRNA is attacked internally by an endonuclease generating 3' and 5' ends accessible to exonucleases enabling quick removal of the resulting decay intermediates.

A major and perhaps the most important degradation pathway in yeast and metazoans however is deadenylation, decapping and 5' to 3' exonucleolytic decay, also known as deadenylation-dependent decay. This is an ordered pathway with each step being activated by and dependent on the previous step. The pathway does not apparently occur in bacteria as there are no known 5' to 3' exonucleases.

Removal of the polyadenylated tail of mRNA initiates this key pathway and, as stated earlier and described further below, polyadenylation is a highly significant means of translational control and stabilises the mRNA molecule for storage until its activation for protein synthesis. Thus polyadenylation is particularly important in mRNA storage in vertebrate oocytes for use during maturation and early embryogenesis. Two deadenylating activities believed to be important in yeast are the poly(A) nuclease complex and the ccr4/caf1 complex (reviewed in Wilusz *et al.*,

2001). The latter is probably a dual-function complex as its components were earlier identified as transcriptional regulators, thereby demonstrating a functional link between transcription and decay. Although not as well characterised deadenylating activity has been identified in metazoan cells such as poly(A) ribonuclease/deadenylating nuclease (PARN/DAN) (Korner and Wahle, 1997).

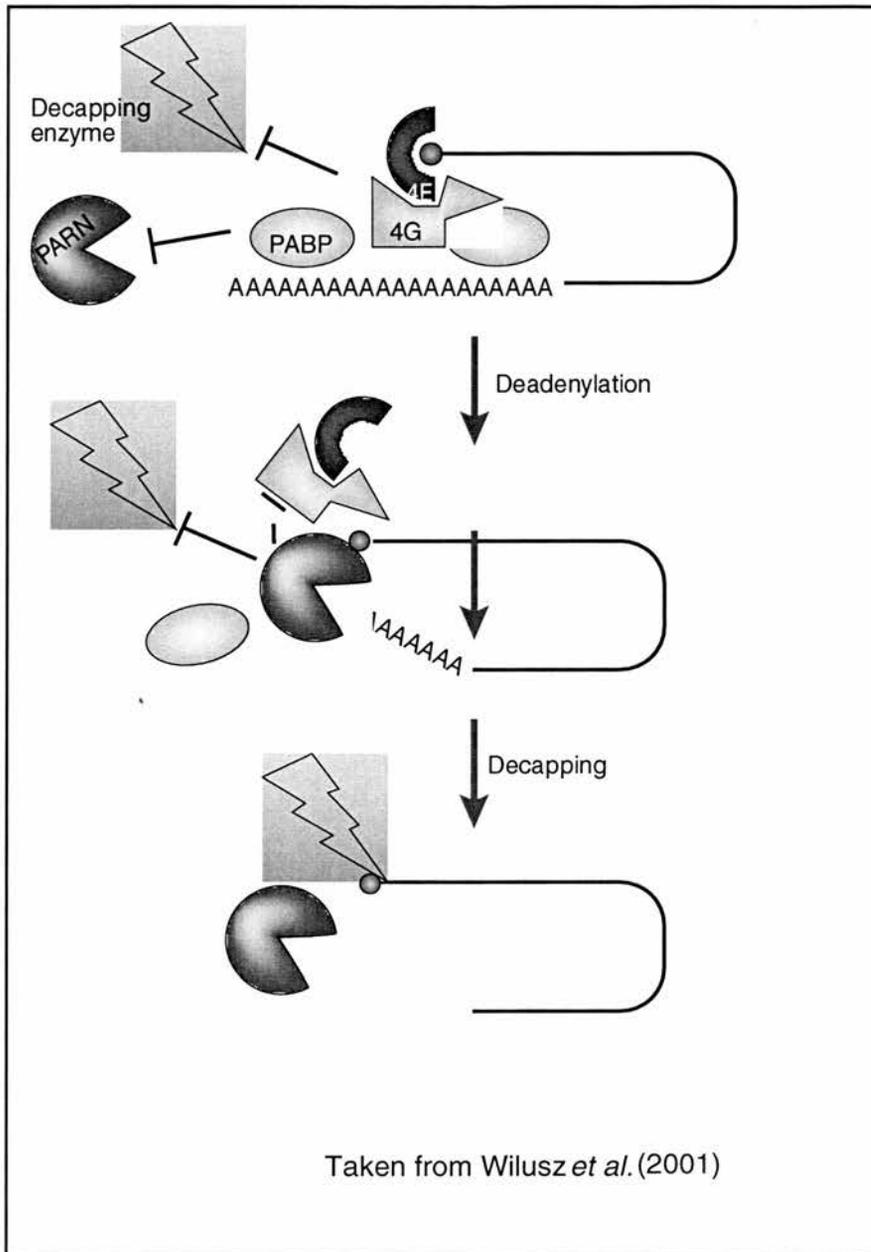


Figure 7: Deadenylation and decapping of mRNA.

The mRNA molecule is understood to be stabilised by circularisation during translation mainly by the interaction between the eIF4E, eIF4G initiation factors and polyA-binding protein (PABP) at opposite ends of the mRNA. eIF4E binds to the 5' mRNA cap structure whilst eIF4G binds to both eIF4E and PABP. Disruption or 'invasion' of the loop is proposed to be carried out by a deadenylase such as PARN from mammals. This is known to have affinity for the cap enabling binding while it deadenylates the 3' end of the mRNA. Further translation initiation is concurrently prevented as is access to the cap by the decapping enzyme. Dissociation of the deadenylase when it has finished its action allows decapping to take over the decay of the mRNA molecule.

As illustrated in figure 7, dissociation of the deadenylase enzyme is thought to allow access to decapping activity such as Dcp1 in yeast. Decapping requires a large complement of additional cofactors and enzymes such as the lsm complex of proteins which have homologues in higher eukaryotes. There does not appear to be a homologue in metazoans of the yeast decapping enzyme Dcp1 although a decapping activity has been demonstrated to exist in mammalian cells (Gao *et al.*, 2001) and homologues of the Dcp1 cofactor Dcp2 and the yeast 5'-3' exoribonuclease (Xrn1) are also known to be present in mammals.

A regulatory factor of Dcp1 has recently been recognised in yeast as Dhh1 which associates in a complex with the yeast 5'-3' exoribonuclease Xrn1 (Fischer and Weis, 2002). Fischer and Weis found that Dhh1 does not operate in nonsense-mediated decay but, rather, in non-aberrant mRNA decay. As Dhh1 is a member of the DEAD-box family of RNA-acting enzymes (usually believed to act as helicases) they propose that it modifies the 5' mRNA structure to facilitate access of the decapping enzyme Dcp1. This therefore could be a factor involved in disrupting the cap-binding complex in deadenylation-dependent mRNA decay as illustrated above. As Fischer and Weis state however, Dhh1 is likely to have further capacity to promote decapping as it can stimulate decapping in the absence of

translation factors.

The circularisation of mRNA by PABP that is believed to occur to promote successive rounds of translation by mRNA stabilisation has been described above. This structure must therefore be perturbed somehow during deadenylation-dependent mRNA decay. Figure 7 illustrates how Wilusz *et al.* postulate that this might occur. Basing their model on the affinity of mammalian poly(A) ribonuclease for the 5' cap of mRNA, they propose that the ribonuclease destabilises both PABP and the 5'-cap from the polyA tail and PABP from the 5' cap thereby preventing decapping until deadenylation is finished. The disruption or 'invasion' of the closed mRNP loop would presumably require a weakening of the interaction between the components of the translation initiation complex.

The above describes a general mechanism of mRNA decay which may occur by random dissociation of the stabilising initiation complex and associated factors but many mRNAs undergo changes in stability in response to external signals which elicit their effect through cellular signalling pathways. This requires the mRNA to be able to respond to such pathways and is achieved through sequence elements most commonly it seems in the 3' UTR of the mRNA molecule. The most intensely studied are A+U rich elements (AREs) and are known to be found in 3' UTRs of

the mRNAs encoding regulatory molecules such as transcription factors, cytokines and growth factors. Certain mRNAs however have stability elements in their 5' UTR such as the mRNA for the cytokine *interleukin-2* which is found in T-cells (of the immune system) and becomes stabilised when the T-cells are activated as part of the body's immune response (Chen *et al.*, 1998). AREs are understood to be mainly responsible for destabilising mRNAs but certain kinds can also promote stabilisation. A great amount of metazoan mRNAs apparently contain AREs as indicated by the diversity of sequence and function of ARE-bearing human mRNAs (Bakheet *et al.*, 2001). Similarly a wide variety of ARE-binding proteins responsible for inducing the stabilisation (*e.g.* HuR) or destabilisation (*e.g.* AUF1) of the mRNA have been identified and are the subject of continued investigation (reviewed in Wilusz *et al.*, 2001).

In view of the proposed model of deadenylation-dependent mRNA decay (figure 7) in which the deadenylase is thought to disrupt the stable loop of mRNP formed by the interaction of PABP with the translation initiation factors, it is conceivable that the ARE-binding proteins alter the affinity of the components at the termini of the mRNA molecule for each other or for the mRNA.

Several examples of specific extracellular signals influencing the decay

rates of mRNAs come from the studied effects of signalling molecules such as hormones and cytokines. An important mRNA stabilising signalling pathway in immune cells is the p38 mitogen-activated protein kinase pathway although Wilusz *et al.* (2001) also point to its similar role influencing non-immune related mRNAs and to the stabilisation brought about by external stimuli which raise intracellular calcium levels.

It has already been stated that polyadenylation, instigated by CPEB, is an important method of translation control in eukaryotes. Polyadenylation is responsible for the initial stabilisation the mRNA molecule and is a particularly important process to vertebrate oocytes which store large amounts of mRNA during oogenesis for mobilisation onto ribosomes in part during maturation and then during early embryogenesis. Such key developmental proteins as Mos are stored in this way as discussed earlier. Cytoplasmic polyadenylation by CPEB involves extension of a pre-existing polyA tail formed in the nucleus and polyadenylation is directed by two sequence elements in the 3'UTR: the polyadenylation hexanucleotide (which also directs initial nuclear polyadenylation of the early mRNA) and the cytoplasmic polyadenylation element (CPE). Much information on the activity of these elements and the associated proteins has been drawn from studies in *Xenopus* oocytes. Prior to maturation,

CPEB binds to the CPE-bearing mRNA (*e.g. mos, cyclin B1*), keeping it in a repressed state by inhibiting translation initiation. The CPEB therefore acts as a masking factor on these mRNAs. Masking is thought to be attained through another protein, maskin, which binds simultaneously to CPEB and the 5' cap-bound initiation factor eIF4E (Stebbins-Boaz *et al.*, 1999). Consequently eIF4G is prevented from binding to eIF4E and the translation initiation complex cannot form.

During oocyte maturation CPEB is phosphorylated by the kinase Eg2 (although its role may not be responsible for initial *mos* mRNA translation as discussed earlier). This phosphorylation enables CPEB to recruit another factor CPSF (cleavage and polyadenylation specificity factor) to the mRNA which binds to the hexanucleotide and in turn recruits polyA polymerase to the 3' terminus of the mRNA (reviewed in Mendez and Richter, 2001). The relief from maskin-mediated translation repression occurs along with polyadenylation and Mendez and Richter propose, based on this observation and on evidence for direct eIF4G and PABP interaction (*e.g. Imtaka et al.*, 1998), that a PABP-eIF4G complex forms which displaces maskin from eIF4E at the 5' mRNA cap and forms a more stable complex thus enabling establishment of the translation initiation complex. That the PABP displaces maskin however remains to be more decisively

demonstrated.

As might be expected given the range of proteins that can bind to mRNA and the sequence variation in the non-translated regions there are a number of further examples of stabilising or destabilising proteins known to bind to the terminal or other regions of mRNA. CPEB-mediated translational control is one of the best characterised though and certainly one of the most important in the early development of *Xenopus* and thus probably other vertebrates. There are also exceptions to the putative general model of deadenylation-dependent mRNA decay.

Two examples of stability regulators operating differently to those described so far include the iron-regulatory protein and the complex of proteins which binds to the mCRD (major protein-coding-region determinant) element found in *c-fos* mRNA. The iron-regulatory protein binds to stem-loop structures in the 3'UTR of *transferrin* mRNA and protects from endonuclease attack when iron levels are low in the cell whereas in high iron concentrations the conformation of the protein is modified reducing its affinity for the mRNA element (reviewed in Klausner *et al.*, 1993). Thus IRP provides an additional level of protection beyond deadenylation-dependent mRNA decay.

The proto-oncogene *c-fos* contains the mCRD which is thought to be a

stabilising element. It is unusual in that it is located within the coding (translated) region of *c-fos* as opposed to the non-translated termini. A set of proteins assemble on the mCRD and activate decay by interaction with the polyA tail, but only during translation. It is proposed that it is the disruption of the complex by the translocating ribosome that causes the complex to initiate deadenylation-dependent decay in contrast to the normal stabilisation that the complex mediates by interaction with the polyA mRNA tail (Grosset *et al.*, 2000).

Given that PABP is understood to stabilise polyadenylated mRNA molecules by interacting with the amino terminus of eIF4G (Imtaka *et al.*, 1998) it is important to note the existence of cap-independent translation initiation which is particularly striking in its prevalence in *Xenopus* oocytes (Keiper and Rhoads, 1997). Keiper and Rhoads reported that the majority of translation in mature *Xenopus* oocytes occurs in a cap-independent manner, that is lacking the normal eIF4G. This raises the question of whether factors other than eIF4G and associated initiation complex components such as PABP are more important with regards to mRNA stability in certain organisms or at certain stages during development. The same authors speculate that mRNAs recruited by polyadenylation during maturation will be more dependent on a cap-

mediated mechanism of translation initiation and another protein may replace the function of eIF4G or even a cleavage product of eIF4G itself. However, it is possible that factors other than PABP would influence the ongoing translation and stability of most *Xenopus* oocyte mRNAs.

Also, as noted by Keiper and Rhoads, it is significant that the translation of many proteins involved in progression through the cell cycle can be increased by injection of the initiation factor eIF4E (which binds to the cap and eIF4G) suggesting that other kinds of genes (*i.e.* non-growth related) are normally dominant in the oocytes which are arrested in the cell cycle at meiosis II. Thus it may be that the oocyte is able to change the dominant type of translation initiation to favour a particular pattern of gene expression. So the possibility of changes in the stability in translating mRNAs at different oogenic stages is a significant factor to consider in this area of investigation.

Importance of RNA secondary structure

At all stages of RNA metabolism — transcription, splicing, nuclear export, translation and decay — proteins are required to protect the structure of the RNA molecule or to modulate its structure for further processing or use as a substrate for packaging or translation proteins. Some of these proteins

have been mentioned above. One commonly encountered secondary structure (characterised by regular folding), particularly in mRNA molecules, is the stem-and-loop represented in figure 8. This represents a double-stranded structure similar to two separate RNA molecules that have aligned by base-pairing but on a smaller scale.

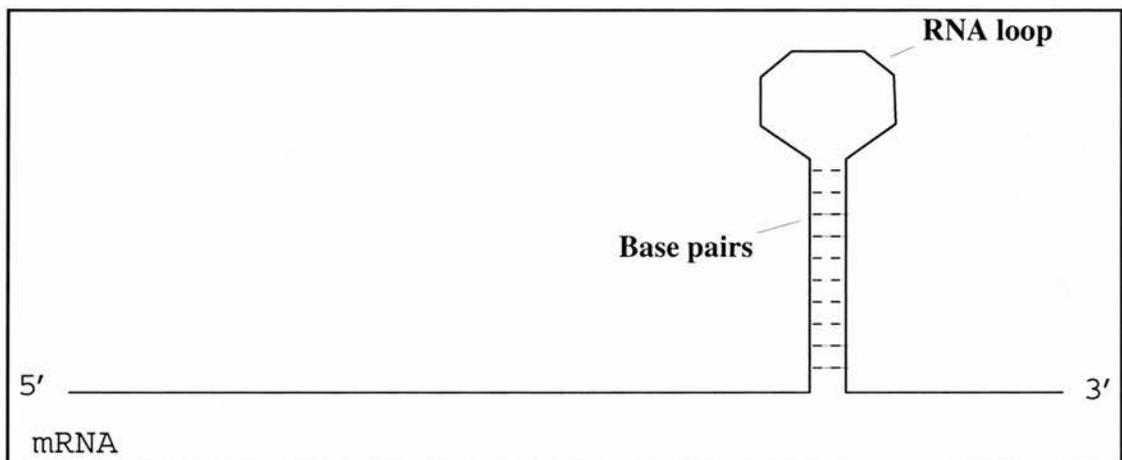


Figure 8: Stem-and-loop secondary structure formed by pairing between complementary bases in a single strand. A single-stranded loop is formed between a helical stem.

RNA molecules may also assume tertiary helical structures depending on their function within the cell or a secondary structure such as the duplex in figure 8 may form unintentionally and need to be removed to avoid impairing the role of the mRNA in the cell.

One major group of proteins involved in unwinding double-strands of RNA is the RNA helicase proteins belonging to the DEAD-box family (reviewed in de la Cruz *et al.*, 1999), so-called because of the DEAD

amino-acid motif common to all members. Most DEAD-box proteins are believed to be involved in RNA metabolism but certain members are known to have roles in separating RNA and protein in ribonucleoprotein complexes and have therefore also been described as RNA-dependent NTPases (using chemical energy from cleavage of nucleoside triphosphates and RNA as a cofactor) or RNPases (reviewed in Schwer, 2001).

Further RNA helicases are found in related families such as the DEAH-box family and all known or suspected RNA helicases have 6 conserved motifs arranged in the same order along the polypeptide chain of the protein. These motifs have been characterised in terms of their functional involvement in ATP binding and hydrolysis and/or RNA binding and hydrolysis. DEAD-box proteins and related families can be described collectively as DExH/D box proteins which covers virtually all RNA helicases as a description of their 2nd of 6 characteristic motifs .

Numerous cellular processes depend upon RNA helicases to remove regions of secondary structure or to make structural modifications within ribonucleoprotein complexes. Based on the structural information available for viral RNA helicase (hepatitis C virus NS3 helicase) and the reduced affinity of the DEAD-box translation initiation factor eIF4A for

single-stranded RNA following ATP hydrolysis, de la Cruz *et al.* (1999) suggest that ATP hydrolysis might open the nucleic-acid binding cleft between two domains of the helicase and translocate the molecule along the ribonucleic acid molecule by a few bases unwinding any duplex in doing so. On binding ATP the cleft would re-close to repeat the cycle thus conceivably being able to make a series of structural modifications in an RNA molecule or to RNA-protein interactions.

Importantly the fact that most DExH/D proteins from *S.cerevisiae* have homologues in mammals underlines their fundamental importance in modifying mRNA structure (and other RNAs) in numerous eukaryotic cellular processes (de la Cruz *et al.*, 1999).

The splicing process that involves the excision of introns from pre-mRNAs in the eukaryotic nucleus seems to be heavily dependent on RNA helicases implying that the maintenance or modification secondary mRNA structure is critical at this time. Splicing requires the assembly of proteins and small nuclear RNAs (snRNAs) into an enzymatic complex known as the spliceosome. The spliceosome subsequently catalyses two consecutive so-called transesterification reactions which cleave the 5' splice site and then cleave the 3' splice site and link the exons respectively. At all stages of spliceosome function, assembly, transesterification and disassembly, RNA

helicases or more accurately DExH/D proteins, are thought to be involved in undertaking the structural modifications to RNA-RNA or RNA-protein interactions.

It is on the basis of the roles of two DExD proteins in dissociating RNA-protein interactions during spliceosome assembly that Schwer (2001) proposes a description as RNPs for these proteins. Sub2p is involved in one structural change which occurs as 2 proteins bound upstream of the 3' splice site (at a region known as the branchpoint sequence) are exchanged for U2 snRNA. The association of U2 snRNP with the mRNA is believed to be stabilised by Sub2p and Kistler and Guthrie (2001) suggest that Sub2p dislodges at least one of the proteins bound at the branchpoint sequence. This is supported to an extent by deletion studies showing that the lack of one of the branchpoint sequence-bound proteins circumvents the need for Sub2p. The protein Prp28p is involved in a similar exchange of factors at the 5' splice-site during spliceosome assembly *i.e.* U1snRNP for U6snRNA. Prp28p is thought to disrupt the U1snRNA/5' splice-site RNA-RNA duplex interaction but mutating the gene for the U1C protein of U1snRNP bypasses the defect of a *Prp28* mutant yeast strain (Chang *et al.*, 1997), raising the question of whether Prp28p interacts with the U1C protein, the RNA or both.

During splicing however there may be several examples of DExH/D protein function where the process is thought to represent a helicase operating on just an RNA substrate. For example Prp5p has been proposed to act upon RNA structure with U2 snRNP to allow it to bind to the branchpoint sequence during the exchange of factors at the 3' splice-site region (O'Day *et al.*, 1996). Therefore it may well be the case that during splicing, some of the proteins that influence RNA secondary structure may have RNA helicase activity and/or RNPase activity.

The role of certain helicases in altering the conformation of mRNA for nuclear export through NPCs and in re-modelling the RNP structures before or after export has been described earlier. This remodelling may be necessary to enable passage through the NPC or to prepare the mRNA for translation releasing shuttling export factors (which can then be re-imported to the nucleus) and allowing translation initiation factors to bind. For example, the DEAD-box RNA helicase Dbp5p is thought to release mRNP from the NPC and from shuttling mRNA export factors (Snay-Hodge *et al.*, 1998). This presumably reflects an RNA or RNA-protein re-modelling process undertaken by the helicase. Although the nature of the precise structural alterations made by the helicase on the mRNA molecules are not known it is clear that the impact of RNA secondary structure is

fundamental to the export process.

Helicases are also believed to be essential for the initiation of eukaryotic translation wherein the 40S ribosomal subunit assembles at the 5' end of the mRNA with the assistance of translation initiation factors one of which is the DEAD-box protein eIF4A. The helicase is believed to be present in order for it to use its helicase abilities to unwind secondary structures in the 5' end of the mRNA facilitating 40S subunit binding or scanning (movement of the 40S-containing pre-initiation complex to the translation initiation codon) thus maintaining translational efficiency (reviewed in de la Cruz *et al.*, 1999). In fact another DEAD-box RNA helicase has been found to be required for translation initiation in yeast although its function does not appear to be superfluous (de la Cruz *et al.*, 1997).

The role of the DEAD-box protein Upf1p in nonsense-mediated mRNA decay has already been described in the context of detecting premature translation termination codons as a member of the surveillance complex that scans the mRNA following translation termination. It is not known whether the protein removes secondary structures during scanning to ensure continuous progress or whether the breakdown of ATP by the protein fuels the scanning process although both activities (helicase and ATPase) are required for the decay process (Czaplinski *et al.*, 1998).

In addition to the deadenylation-dependent mRNA decay that degrades non-aberrant mRNA as part of a functioning, efficient yeast (and presumably higher eukaryotic) cell there is also the capability to fully degrade mRNAs in the 3'-5' direction. An enzymatic complex known as the exosome is believed to be responsible for this pathway (Anderson and Parker, 1998). One DExH-family RNA helicase, Ski2p, associates with the exosome for 3'-5' mRNA decay in the cytoplasm in yeast. Ski2p is a homologue of Dobp, a protein that, similarly, is thought to associate with the exosome in the nucleus in the processing of rRNA precursors. Both proteins may prevent stalling of the exosome as it moves in the 3' to 5' direction on nuclear RNA or cytoplasmic mRNA (de la Cruz *et al.*, 1999) drawing comparisons to the potential role of Upf1p in the surveillance complex following translation termination.

The diversity of function of putative helicase DExH/D proteins reveals their value to the functioning of a eukaryotic cell. Despite the conservation in the motifs of the so-called core-regions the helicases carry out a wide range of distinct cellular functions and the specificity is thought to be determined by less well conserved N-terminal and C-terminal regions by carrying protein- or RNA-binding domains or motifs that direct its subcellular localisation.

The secondary structure of an RNA molecule is used by many proteins as a means of recognition for attachment. These proteins can be diverse in function including proteins within snRNP particles, masking proteins (mRNA storage / translation repression) binding to mRNA and mRNA localisation proteins. In such cases the presence of a particular structure is often more important than the ribonucleotide sequence.

The *Drosophila* mRNA *bicoid* is synthesised in nurse cells but localised by entrapment after transport into the oocyte. The transport into the oocyte is permitted by a sequence-defined localisation element and two stem-loop structures. The subsequent anchoring of the mRNA at the anterior of the oocyte to ensure effective localisation requires another stem loop structure recognised by the protein Staufen (Ferrandon *et al.*, 1994). Bicoid protein is essential in establishing later gradients in gene expression that drive later body patterning.

The yeast mRNA *ASH1* contains a structure-based mRNA localisation sequence that straddles the coding region and the 3' UTR and is recognised by the protein She2. The mRNA is subsequently localised to daughter yeast cells.

Staufen protein contains several double-stranded RNA-binding domains enabling it to bind various mRNAs with a secondary structure but

additional factors may be required for selective mRNA binding (Ferrandon *et al.*, 1994). She2 protein is able to recognise multiple secondary structures in *ASH1* mRNA but using distinct RNA-binding domains to Staufen (reviewed in Jansen, 2001). The presence of double-stranded RNA-recognising localisation proteins in *Drosophila* and mammals indicates the general importance of mRNA secondary structure in eukaryotic mRNA localisation and translation.

The protein components of maternal mRNP in *Xenopus*

The process of *Xenopus laevis* oocyte maturation and the large-scale accumulation of messenger ribonucleoprotein particles (mRNP) for translation at maturation and embryogenesis have been described above. Also the role of mRNP proteins such as CPEB and maskin in repressing the translation of mRNA has been described. However in *Xenopus*, there are 4 highly abundant and well studied components of mRNP particles in addition. These proteins show an estimated mass range of 50-60KDa by SDS-PAGE and consist of the mRNA masking (translation repression) proteins FRGY2a and FRGY2b, the DEAD-box RNA helicase p54 and p52, a protein which may represent a form of the protein RAP55 discovered by Lieb *et al.* (1998).

FRGY2a and FRGY2b have been referred to also as mRNP4/pp60 and mRNP3/pp56 respectively and may represent pseudoalleles — two forms of the same gene borne on the two copies of each chromosome set inherited from each *Xenopus* parent (giving *Xenopus* its tetraploid genome). They are thus usually referred to in the singular FRGY2a/b. The description ascribed to FRGY2a/b as a masking protein comes from the discovery that it is able to repress the translation of specific mRNAs when bound *in vitro* (Richter and Smith, 1984). The name refers to the protein's structural classification as a Y-box protein.

The mRNA masking proteins present within *Xenopus* mRNP particles belong to a particular class of proteins within the OB (oligosaccharide/oligonucleotide-binding) fold superfamily which contain a so-called cold-shock domain (CSD) which bears evolutionary similarity to a domain found in bacterial cold-shock proteins (reviewed in Murzin, 1993). Y-box proteins are a sub-group of CSD proteins involved in single-stranded DNA or RNA-binding and can be found throughout unicellular and multicellular life. Their structure is notable for being modular but a comparison of the structures found in vertebrate, invertebrate, plant and bacterial Y-box proteins reveals the common CSD region (Sommerville and Lodomery, 1996). Those Y-box proteins found in mRNP particles in *Xenopus* belong

to a type seen also in other vertebrates and contain an alanine / proline region proximal to the CSD and alternating regions of basic/aromatic and acidic charge in the extensive C-terminal region. The CSD has a β -sheet structure containing 5 antiparallel β -strands forming a barrel structure.

The Y-box structure permits both ssDNA and RNA binding through features such as aromatic residues in the CSD and the positively charged basic residues in the C-terminal region. These would allow stacking with nucleotides and charge interaction with the negatively-charged DNA / RNA sugar-phosphate backbone respectively.

In the mRNP particles of *Xenopus*, the Y-box protein FRGY2 has been well characterised and is capable of displaying selectivity in binding to RNA sequences containing a particular consensus sequence via its CSD (Bouvet *et al.*, 1995) but must be able to bind many RNA sequences as indicated by its wide scale presence within mRNP particles in the oocyte — comprising up to an estimated 5% of the total oocyte mass in the early stages of oogenesis (Sommerville and Ladomery, 1996). However, the work of Bouvet *et al.* also showed that the C- and N-terminal domains appear to enhance RNA binding through stabilisation of the CSD binding, indicating the importance of regions other than the CSD.

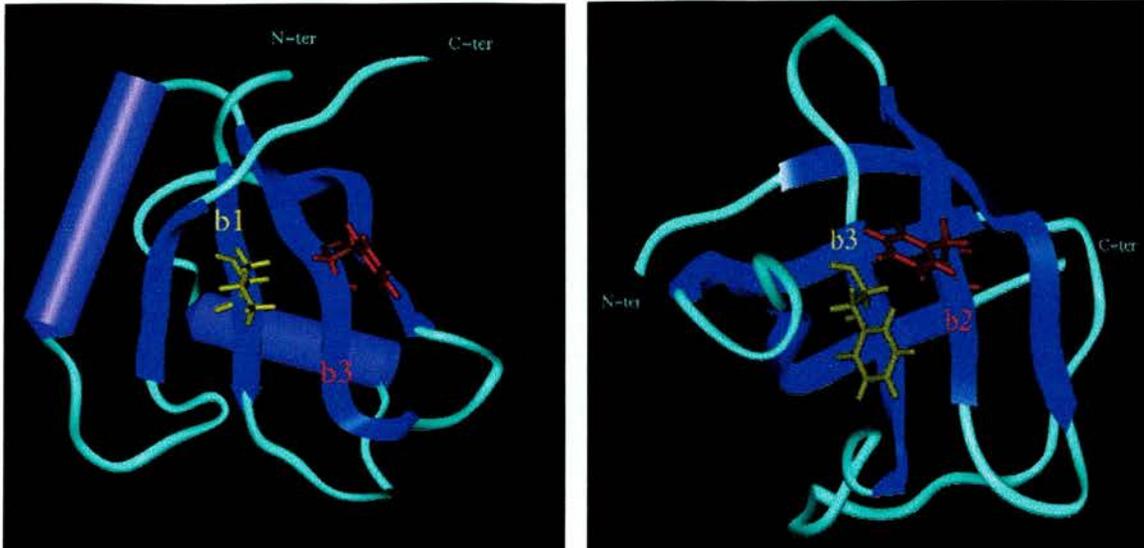
Structural studies have revealed similarities in both sequence and structure

between different RNA binding proteins which may represent either conservation of sequence or convergent evolution towards a common structural binding strategy. FRGY2 for instance bears two RNA-binding peptide motifs within its CSD called RNP1 and RNP2 which are conserved in the RNA recognition motif/RNA-binding domain (RRM/RBD) found in proteins such as nucleolin (Ginisty *et al.*, 1999) and U1A protein from the U1A snRNP (Hoffman *et al.*, 1991).

The structural comparisons of Manival *et al.* (2001) revealed how the similarities between nucleolin's third RRM and FRGY2's CSD conferred similar ways of interacting with single stranded RNA and also the common action of their auxiliary domains, namely 4 basic/aromatic 'islands' in FRGY2.

Both the RRM domain and the CSD bind to the nucleic acid through a beta-sheet structure consisting of antiparallel (running in opposite directions) strands arranged adjacent to each other. The RNP1 and RNP2 motifs are located on the 1st and 3rd strands of a $\beta\alpha\beta\beta\alpha\beta$ β -sheet structure in a RRM domain whereas the two motifs are located on the second and third β -strands respectively of a $\beta\beta\beta\beta\beta$ β -sheet structure within FRGY2 (Manival *et al.*, 2001). Superimposition of structural models of the third RRM of nucleolin and the CSD of FRGY2 (figure 9) by Mainval *et al.*

(2001) indicated close spatial proximity of the RNP1 motif to the RNP2 motif in both proteins despite the structural and overall sequence differences.



RRM

FRGY2 CSD

Figure 9: Structural models of the third RNA-recognition motif of nucleolin and the cold-shock domain of FRGY2.

The third RRM of nucleolin has a structure $\beta\alpha\beta\beta\alpha\beta$, alpha-helices coloured purple and β -strands coloured blue. The RNP1 motif is located on the β 3-strand and the side-chain for a phenylalanine residue conserved amongst RRMs is shown. The RNP2 motif is located on the β 1 strand and a conserved valine motif side-chain is shown.

FRGY2 has a $\beta\beta\beta\beta\beta$ structure and the β -strands are shown. The RNP1 motif is on the β 2-strand and a side-chain for a conserved phenylalanine residue is shown. The RNP2 motif is located on the β 3-strand and another conserved phenylalanine residue side-chain is shown.

Diagram adapted from Manival *et al.* (2001).

Manival *et al.* (2001) also speculate on the possibility of the CSD domain of FRGY2 being responsible for multimerisation of the molecule and hypothesize that this may cause the packaging which is observed in mRNP particles in *Xenopus* oocytes.

The C-terminal 'auxiliary' domain of FRGY2 containing the repeated basic/aromatic 'islands' is able to bind RNA sequences apparently non-specifically (Bouvet *et al.*, 1995) but Matsumoto *et al.* (1996) showed that this domain is essential for translation repression by FRGY2 whereas the CSD merely enhances the binding. The two functional domains of FRGY2 thus seem to cooperate to link packaging and translation repression of maternal RNA in the formation of mRNP particles within the nucleus. FRGY2 is phosphorylated extensively within mRNP at casein kinase II (CK2) phosphorylation sites which is thought to stabilise the masking of mRNA within the particle (Sommerville, 1999). Indeed, the catalytic subunits of this enzyme are thought to be a stable component of *Xenopus* oocyte mRNP particles (Cummings and Sommerville, 1988). Comparing by electrophoresis the peptides generated by proteolysis of purified FRGY2 phosphorylated *in vivo* and by CK2 *in vitro* reveals identical patterns suggesting CK2 is the enzyme responsible for the phosphorylation of FRGY2 in oocytes (Deschamps *et al.*, 1997). The

masking and unmasking of maternal mRNA in *Xenopus* oocytes is thought to be co-ordinated process involving Y-box proteins, the RNA helicase p54 and a protein-kinase presumably CK2. This process is discussed below with emphasis on the function of each member of the masking complex. CK2 phosphorylation sites are mainly located within the acidic regions of the FRGY2 molecule . The structure of the protein is represented in figure 10. From the diagram it can be seen that the structure of FRGY2 is modular consisting of acidic regions rich in aspartic acid and glutamic acid residues and basic residues rich in arginine. The basic regions also contain aromatic residues (phenylalanine and tyrosine) giving then the name basic/ aromatic islands.

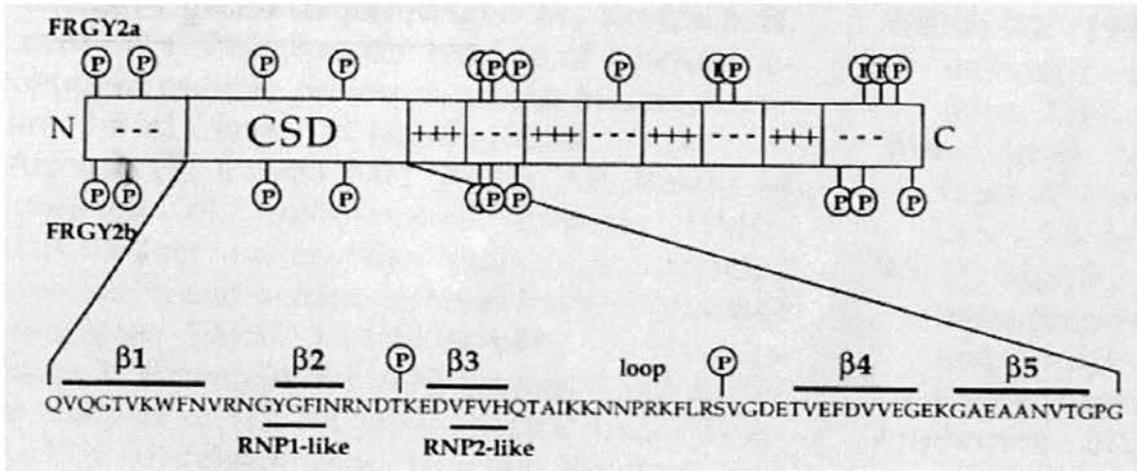


Figure 10: Structure of FRGY2.

The CSD and surrounding acidic (-) or basic/aromatic (+) modules are shown. The magnification of the CSD shows the 5 β -stranded structure and the RNP motifs. CK2 phosphorylation (P) consensus sites are also shown and are mainly sited within the acidic regions. Diagram adapted from Sommerville and Lodomery (1996).

As stated CK2 is thought to be responsible for the phosphorylation of FRGY2 and stabilisation of mRNA masking in mRNP particles. The same enzyme is also thought to associate with the particle in the nucleus and stabilise the translation repression by FRGY2 as co-injection of reporter mRNA and CK2 inhibitors results in higher translation of the reporter than when the reporter is injected alone into the cytoplasm or when the reporter is injected into the nucleus (Braddock *et al.*, 1994). Thus the repression process seems to begin in the nucleus with the combined action of various factors on the mRNA, an environment which does not apparently exist to the same effect in the cytoplasm (reviewed in Sommerville and Lodomery, 1996).

Treatment of oocytes with progesterone reverses the translational repression of nuclear-injected RNA and this effect is itself negated by simultaneous treatment with a phosphatase inhibitor (Braddock et al., 1994) suggesting that FRGY2 de-phosphorylation may be a component of the recruitment of mRNAs (*i.e.* unmasking of mRNAs) occurring during oocyte maturation. The study of Braddock *et al.* also showed how the presence of an intron within the mRNA injected inhibits masking. This could indicate that the masking of a transcript occurs in the absence of splicing or that FRGY2 favourably selects those nascent transcripts which are from intron-less genes of which there are several examples such as *mos* and certain histone transcripts. However it is not certain what proportion of spliced mRNAs remain unmasked relative to unspliced mRNAs only that all masked maternal mRNAs analysed prior to this study originate from intron-less genes (John Sommerville, pers comm.). There is however evidence from immunostaining that FRGY2 is present in the vicinity of the lateral loops of *Xenopus* 'lampbrush' chromosomes in immature oocytes indicating the point of masking protein binding (Sommerville and Ladomery, 1996).

The RNA helicase p54 was identified as an abundant DEAD-box containing ATP-dependent component of mRNP particles in *Xenopus* by

Ladomery *et al.* (1997). The study revealed Xp54 as a member of the DEAD-box protein family. Orthologues (from genes homologous between different species but with there being a single most recent common ancestor as opposed to deriving from a duplicated gene) include Me31B in *Drosophila melanogaster*, Dhh1 in budding yeast (*Saccharomyces cerevisiae*), CGH-1 in *Caenorhabditis elegans*, Ste13 in fission yeast (*Schizosaccharomyces pombe*) and p54/RCK in humans.

Computer predictions yield high degrees of similarity between the orthologues as indicated by the boxed regions in figure 11, indicating conservation of sequence in this particular helicase between different organisms. As expected the RNA helicase core domains are well-conserved in general but there is less conservation amongst the N- and C-termini which relates to the proposed functions mentioned above of specifying respective RNA targets or other proteins with which to interact according to the organism. An analysis of the degree of residue conservation within the helicase region in p54 orthologues by Navarro *et al.* (2001) revealed 70% identity between CGH-1 and Ste13, Dhh1, Me31B or RCK whereas comparison with other RNA helicases such as human eIF4A (translation initiation factor) gave a much lower degree of amino acid similarity. As the authors state, this indicates that the

conservation between the p54 orthologues is for reasons other than the helicase function alone. Additionally Ladomery *et al.* (1997) found a 94% amino acid similarity between Xp54 and human RCK. This in turn perhaps reinforces the specialisation of function in this group of proteins.



Figure 11: Alignment of the DEAD-box RNA helicase subfamily including p54. Organisms from which named proteins derive are specified in the text. Details of the alignment protocol and EMBL protein databank accession numbers are given in Ladomery *et al.* (1997).

The work of Ladomery *et al.* (1997) also revealed the expression pattern of *Xenopus p54* (*Xp54*) mRNA and protein. *Xp54* mRNA is maximally

expressed during oocyte stages I and II then sharply declines until it is undetectable at early embryogenesis. This is thought to be because early oogenesis witnesses the greatest production of proteins for mRNP and the requirement for p54 protein may decline after this period. The protein is also expressed at its highest level early in oogenesis but remains at this level until the blastula stage of the embryo and then declines but is still detectable at the early tadpole stages of development. In addition a 54KDa (kiloDalton) protein can be detected in adult somatic tissues with antibodies raised against the oocyte form of the protein although the *Xp54* transcript could not be detected in adult tissues. This indicates that a similar somatic protein exists yet with notable difference at the genetic level to the maternal version.

The function of *Xp54* remains to be more fully defined but it can be speculated upon using both evidence from work on the protein itself such as that described above and from information gained on the function of *Xp54* orthologues. As a component of mRNP particles p54 could be involved in assisting translation initiation (like eIF4A) or elongation by removing secondary structures in the mRNA at times when stored maternal mRNA is mobilised onto polysomes (structure constituting multiple ribosome transits along an mRNA molecule) *i.e.* oocyte

maturation and early embryogenesis. The most closely related homologue to CGH-1 in *C.elegans* is eIF4A which supports a role in translation initiation (Navarro *et al.*, 2001).

Xp54 might in addition or instead function in the nucleus in the formation of mRNP particles removing secondary structure to assist the binding of other mRNP proteins such as the Y-box masking proteins. Xp54 is believed to accompany mRNA molecules in their transit from the point of transcription at the chromosome to the cytoplasm within transcribing oocytes (Smillie and Sommerville, 2002) by an unidentified export pathway. Furthermore when the oocyte is not transcriptionally active the subcellular distribution of Xp54 is mainly cytoplasmic which is maintained by virtue of a signal or signals within the protein's C-terminus. Its import into the nucleus apparently also relies upon an N-terminal region and during transcriptionally silent periods the protein is exported from the nucleus by means of a leucine-rich NES which is the same pathway as the human immunodeficiency virus (HIV) protein REV, that is by means of the CRM1 export receptor (involved in snRNA nuclear export, Ohno *et al.*, 2002) as may be expected from its sequence similarity to REV. On activation of transcription Xp54 moves to the nucleus having being released from the cytoplasmic retention. Thus Xp54 may well have more

than just a cytoplasmic role and so may be doing more than just binding mRNA in the nucleus in preparation for a later, cytoplasmic role in translation initiation.

That Xp54 bears a REV-protein like NES is not apparently related to mRNA export as Smillie and Sommerville (2002) did not find evidence for CRM1 export of mRNP by p54 and CRM1 has been refuted to be a significant export means for mRNA (Neville and Rosbash, 1999). Its significance may be as a mechanism to remove excess Xp54 from the nucleus of transcribing oocytes.

A review of recent research into Xp54 orthologues reveals that similarities, often fundamental, are increasingly being drawn between the most members of this group of RNA helicases. The most crucial similarity it seems is to ensure appropriate translation of certain mRNAs during meiosis so that the gamete (oocyte or sperm cell) develops normally and is viable *i.e.* capable of forming an embryo with a gamete of the opposite sex.

In *Drosophila* for example the Xp54 orthologue Me31B appears to be required to repress the translation of certain mRNAs as they are transported to the oocyte during oogenesis from the surrounding nurse cells in the animal's egg chamber (Nakamura *et al.*, 2001). This transport is

necessary to provide the oocyte with genetic messages that can subsequently become localised asymmetrically within the oocyte and generate protein gradients. These gradients in turn cause the development of the required body pattern of the embryo. As stated earlier, Me31B forms mRNP particles with a protein, Exuperantia, that is required for localisation of certain mRNAs that are vital for the normal development of the fertilised oocyte. Some of the mRNAs encoding determinants of embryonic pattern formation, such as *oskar* mRNA, when translated prematurely are lethal to the embryo. Thus Me31B seems to prevent such mRNAs from being inappropriately expressed during oogenesis and generating unviable oocytes. Nakamura *et al.* also suggest that Me31B could also antagonize the function of the closely related translation initiation factor eIF4A to repress rather than initiate translation. Additionally Me31B-containing particles also contain a Y-box protein, Yps, recently implicated in translational control and localisation of *oskar* mRNA (Mansfield *et al.*, 2002).

The *C.elegans* Xp54 orthologue was identified by Navarro *et al.* (2001) as an essential factor for normal progression through meiosis in sperm and egg formation. A lack of CGH-1 triggers a physiological apoptosis (*i.e.* a distinct apoptosis mechanism, involving MAPK signalling) in oocytes

during meiosis I prophase and sterile mature sperm. The authors suggest that CGH-1 may also be involved in regulating the translation of stored mRNAs as the protein is found in P granules (RNA-protein granules involved in germline specification and development) and somatic (not germline associated) granules that are present until the early embryo stages and the protein disappears at approximately the same time as maternal mRNAs in the embryo. Thus physiological apoptosis may be triggered by mis-regulation of the translation of maternal mRNAs as a result of the absence of CGH-1. The possibility of a role in regulation of mRNAs stored as mRNP is furthermore shared therefore between Me31B, CGH-1, Xp54 and also mouse p54 which associates with a Y-box protein in maternal mRNP particles (Paynton, 1998).

The orthologue produced by fission yeast *Schizosaccharomyces pombe*, Ste13, is also necessary for the progression into meiosis which occurs as the yeast cell exits from the mitotic cell cycle upon nitrogen starvation. Individuals which lack the Me31B gene in *Drosophila* exhibit collapsed egg chambers and are incapable of completing oogenesis (Nakamura *et al.*, 2001) giving all of the Xp54 orthologues in *C.elegans*, *Drosophila* and *S.pombe* an essential role in gametogenesis.

A more specific role has been identified for the Xp54 orthologue in

Saccharomyces cerevisiae, Dhh1, which as described above was found to be required for efficient decapping by the decapping enzyme Dcp1, following mRNA deadenylation and was suggested to promote access of Dcp1 by modifying 5' mRNA structure (Fischer and Weis, 2002). A role at the 5' end of the mRNA molecule draws comparison to the role suggested for Xp54 by Ladomery *et al.* (1997) in promoting translation initiation. Assuming a dual role for Xp54, in the nucleus (assisting the packaging of mRNA into Y-box protein-containing mRNP particles) and the cytoplasm (facilitating translation initiation and/or elongation), the activity of the helicase must experience a change in regulation. In other words it may change from being a component of a translational repression particle to a contributor towards translational initiation. As mentioned above FRGY2 is phosphorylated extensively in mRNP particles presumably by CK2 in a way that seems to increase mRNP stability and thus efficacy of translational repression (Sommerville, 1999). However, it is less clear whether Xp54 is the subject of early CK2 regulation since despite the presence of multiple consensus CK2 phosphorylation sites within its primary sequence the protein's phosphorylation is less extensive in early stage oocytes when transcription is maximal (Cummings and Sommerville, 1988). Such regulation would be in the form of dephosphorylation or CK2

phosphorylation to activate the helicase when its activity was required.

Alternatively, an auxiliary protein factor or a coenzyme may be required for the onset of Xp54 helicase activity.

One potential candidate for the role of Xp54 cofactor has been identified (and had its cDNA sequence cloned) in the related amphibian, the salamander *Pleurodeles waltl* (Lieb *et al.*, 1998). This protein is named RAP55 (RNA-associated protein 55) after its predicted molecular mass of 55KDa and can be isolated from polyadenylated mRNP and resolved by SDS-PAGE as can Xp54. It was initially discovered accidentally as a product of a *Pleurodeles* cDNA library which reacted with an antibody that also reacted with proteins associated with oocyte lateral chromosomal loops (although Lieb *et al.* could not detect the RAP55 in the nucleus subsequently). Also it expressed in *Pleurodeles* in oocytes, eggs and the early embryo in common with p54 in *Xenopus*. The most abundant protein components of poly(A)+ mRNP particles in *Xenopus* have masses of around 50-52KDa, 54Da, 56KDa and around 59-60KDa apparent by SDS-PAGE. However the apparent mass of RAP55 synthesised from cDNA by *in vitro* translation is 68KDa in *Pleurodeles* protein extracts. Antibodies raised in guinea pigs against purified RAP55 cross-react on immunoblots with proteins of apparent mass of 140KDa and 56KDa and the same three

protein bands are revealed by the same antibody in *Xenopus* oocyte and embryo extracts although RAP55 declines during early embryogenesis and p140 is only found in oocytes. Only RAP55 and p56 seem to be components of mRNP particles. Unpublished data also shows the same antibody reacting on immunoblots of *Xenopus* oocyte extracts with a protein of apparent mass of 52KDa (J.Sommerville pers comm.). Whether this protein, one of the 4 most abundant proteins found in *Xenopus* oocyte mRNP particles indeed represents RAP55 remains to be confirmed.

Additionally the situation is perhaps even further complicated by the SDS-PAGE resolution of a 68KDa protein from poly(A)+ mRNP particles which was discovered to be the only other protein along with p54 which could be crosslinked to ATP (Ladomery *et al.*, 1997).

In contrast to the FRGY2 Y-box proteins, neither p56 nor RAP55 could be crosslinked to mRNA by photo-crosslinking (Lieb *et al.*, 1998) suggesting there isn't a direct permanent interaction with the mRNA within mRNP.

Whether p54 binds directly to the repressed mRNA is not known only that the association of p54 with the mRNP appears to be due to relatively weak charge interactions (Ladomery *et al.*, 1997). The presence of RAP55 within amphibian maternal mRNP particles and the gradual disappearance following fertilisation strongly suggests that the protein has a function in

translational control of maternal mRNP.

Masking and unmasking of maternal mRNA

As stated above, it has been proposed that the RNA helicase may function in the assembly of mRNP particles and the activation of the genetic message of the transcript stored within. These functions are examined more closely below.

The transcription pattern of the *Xp54* message was demonstrated to correspond to the high level of transcription normally observed in early oocytes with a decline in the level of *Xp54* mRNA after these stages. The corresponding protein was also demonstrated to be present from the earliest defined oocyte stage (stage 1) with a decline in cellular levels from the blastula stage. Thus, *Xp54* would seem to be a fundamental component in the functioning of mRNP particles being available for this from the outset of mRNP activation during maturation.

Sommerville (1999) provides an example to describe how an RNA helicase could have an essential presence in conjunction with Y-box proteins within mRNP particles and so have relevance to *Xp54* and *FRGY2* in *Xenopus* mRNP particles. In this model, the bacterial cold-shock protein *CspA* is referred to which maintains RNA in a single-

stranded conformation at sub-optimal growth temperatures which can favour stable but aberrant intra- or inter-strand RNA duplexes which would hinder the passage of a ribosome along the mRNA molecule during translation. A related protein has also been identified, CsdA (Jones *et al.*, (1996), with helicase activity which is postulated to work alongside CspA, removing duplexes which would adversely affect translation when it is required. Thus, the translation-inhibiting effects of reduced temperature are minimised along with the loss in efficiency of translation.

Similarly, in *Xenopus* it is possible that Xp54 works in conjunction with FRGY2 and other proteins in the mRNP particle to regulate mRNA conformation. However, as stated the early expression of Xp54 at a time when transcripts are rapidly being produced suggests that Xp54 could possibly function in the assembly of mRNP particles in addition to facilitating translation and improving translation efficiency. It is envisaged that Xp54 would unwind duplexes in the newly transcribed RNA enabling the binding of mRNP proteins such as FRGY2 (Ladomery *et al.*, 1997; Sommerville, 1999). Thus, analogies may be drawn with Xp54 and FRGY2, the separate RNA-related activities being carried out by different proteins.

The likelihood of Xp54 functioning as an essential factor in the mRNP

assembly process is supported by the early presence of the protein in the oocyte and the ability in common with eukaryotic CSD proteins to bind single-stranded nucleic acids (Sommerville and Lodomery, 1996). In addition, Xp54 —although predominantly cytoplasmic — can be detected in the nucleus in smaller amounts in the localisation of chromatin for example (Smillie and Sommerville, 2002) suggesting a role for Xp54 at an early stage in the mRNP metabolism. Thus a role in maintaining RNA stability and repressing translation in addition to a translation recruitment role would also explain the abundant presence of Xp54 in mRNP particles. Regardless of whether Xp54 has a general function in mRNP assembly or release of mRNA from its masked state into polysomes for translation or both, a mechanism or number of mechanisms is required to ensure activation of the helicase at appropriate times. Perhaps the most obvious way by which this might be achieved is through the mRNP-associated protein kinase CK2 .

The phosphorylation of the masking protein with which CK2 is associated, FRGY2, has been proposed to be responsible for enhancing its stability of binding with mRNA in a model for non-productive translation (Sommerville and Lodomery, 1996). The presence and the apparent importance of CK2 in regulating the masking process makes regulatory

interactions with Xp54 a possibility. This is reinforced by the presence of 4 potential CK2 phosphorylation sites in the C-terminal domain of Xp54 and the deduction that Xp54 phosphorylation occurs during a time coincident with mobilisation of mRNA within associated particles onto ribosomes for translation, *i.e.* early oocyte maturation (John Sommerville, pers. comm.). This phosphorylation-dependent activity in Xp54 however would require that the helicase was phosphorylated at a time after mRNP assembly given that FRGY2 phosphorylation drives assembly and masking of the message. Ladomery *et al.* (1997) suggest that the putative CK2 sites in Xp54 (highlighted in figure 12) may be blocked during mRNP assembly, gaining the the ability to receive phosphates later in maturation. The same authors mention the possibility of the involvement of another factor which can only act upon Xp54 at a certain stage during maturation perhaps depending partially or entirely on its phosphorylation state.

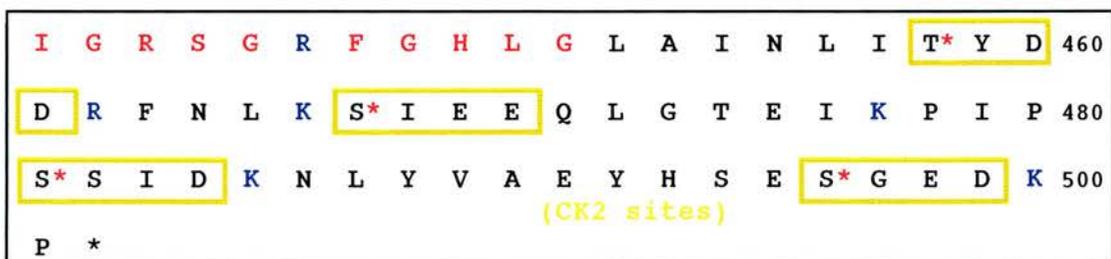


Figure 12: Position of CKII consensus phosphorylation motifs (boxed) within the amino acid sequence at the C-terminus of Xp54.

Regardless of various, more specific mechanisms of translational activation such as CPEB-associated polyadenylation of certain mRNAs, the general abundance of the various components of mRNP particles in maturing oocytes such as those of *Xenopus* implies that a general mechanism of translational activation exists additionally. The interaction and regulation of major components such as FRGY2 and Xp54 RNA helicase would seem to be fundamental to this.

Objectives

The aim of this study is to further investigate the activities of Xp54 in its role as a major component of mRNP particles in translational control during *Xenopus* oogenesis and oocyte maturation. Specifically, the role of the protein in the nuclear export or stability of maternal messages (those mRNAs formed before fertilisation) is investigated. Overexpression of the cloned mRNP protein or knockout of the protein's translation using antisense morpholinos is used to examine the effect on levels of specific maternal mRNAs and also the effects on general mRNA stability in order to attempt to deduce the normal roles of this important protein and the scope of its functioning in early development. Also, investigations are carried out into how the action of two other major mRNP particle

components, FRGY2 and p52, may relate to Xp54's influence on mRNA.

Methods and Materials

Media

Bacteria

Bacteria were cultured in Luria broth (LB) medium. For plating of bacteria, sterilised medium containing 1.5% agar was poured onto plates after addition of ampicillin/carbenicillin to 50µg /ml. The same concentration of ampicillin was used in liquid cultures.

LB Medium

10g/l peptone

5g/l yeast extract

10g/l NaCl

Cold storage of bacterial culture

A volume of 0.9ml of bacterial culture grown overnight at 30°C in LB broth was transferred to a vial together with 0.1ml of glycerol as a cryopreservant and the mixture stored at -70°C. Samples of culture were recovered using a flame-sterilised wire loop to plunge into the frozen stock to detach a fragment which was then streaked onto a selective nutrient agar plate.

Oocytes

Oocytes were maintained in OR2 buffer as described below.

Bacterial strains

The following bacterial strains were used during this study.

Novablue (Novagen): An *E.coli* K12 strain with high transformation efficiency. *recA* and *endA* mutations giving high yield of plasmid DNA due to disruption of the respective recombination and endonuclease enzymes.

Oocyte preparation, injection and extraction for SDS-PAGE

Preparation of dissected ovaries

Approximately 5ml of dissected ovary was incubated with 8ml OR2 buffer containing 0.2% collagenase for 1½ hours in order to remove follicle tissue. Then, 1mM CaCl₂ was added to a separate tube of 50ml OR2 buffer which was used to wash the oocytes in order to inhibit further collagenase action. Before the final wash, antibiotic / anti-mycotic mixture was added to the OR2 buffer to 0.5% v/v to inhibit fungal and bacterial growth. Oocytes can be maintained in such buffer for several days at room temperature before the onset of oocyte disintegration.

OR2 Buffer	g/litre
NaCl	5.05
KCl	0.185
CaCl ₂	Omitted until after collagenase treatment
MgCl ₂	0.203
Na ₂ HPO ₄	0.358
HEPES	1.192
Polyvinylpyrrolidon	0.500
NaOH (3.8mM)	to pH 7.8

Oocyte injection

Oocytes were transferred onto a 3ml petri dish lined with a gauze grid and containing OR2 buffer to enable orientation for injection of nucleic acids or proteins. Animal poles were positioned upwards for nuclear injection and downwards (vegetal pole face-up) for cytoplasmic injection. After positioning, buffer was removed and the petri dish was transferred under a microscope for viewing of microinjection with an appropriately positioned, adjustable glass needle.

For nuclear injection, a total of about 2µl DNA or protein solution was placed on plastic film for uptake into the glass needle and each nucleus received approximately 10nl of nucleic acid/protein or other solution.

Quantities of DNA or protein varied according to the procedure used for their preparation and are stated in Results where relevant. This was achieved by adjusting the needle pressure so that a continual, steady stream of solution was expelled as the oocytes were injected at a brisk, regular pace.

Approximately 20nl of nucleic acid/protein or other solution was injected into the cytoplasm of oocytes again at a brisk pace and with a corresponding increase in the amount of total solution uptaken beforehand with the glass needle and increase in injection duration.

Nucleus / cytoplasm extraction

In order to prepare extracts of whole oocytes or oocyte compartments for further analysis, oocytes were placed on 3M filter paper-lined 3ml petri dishes and covered with mineral oil, animal pole (dark pigmented face) face-upwards. A tear was then made on the animal pole to allow the clear nucleus to emerge which was then transferred with an automatic pipette to a vial containing, unless stated, 20µl water. If necessary nuclei were washed in mineral oil prior to further processing to remove yolk from the ooplasm. The cytoplasm was subsequently transferred using a pipette to a vial containing, unless stated, 80µl water. Vials containing extracts were immediately and briefly centrifuged then frozen at -20°C for temporary storage or -70°C for longer-term storage. Oocytes to be used for whole protein extracts were transferred, following the particular experimental treatment, into vials and frozen.

Preparation of extracts for SDS-PAGE

In order to prepare the nuclear or cytoplasmic extracts for SDS-PAGE (see below), the layer of remaining isolation oil was first removed. Whole oocytes or cytoplasmic oocytes were then homogenised by pipetting up and down and then a volume approximating the total volume occupied by the extracts of freon (1,1,2-trichlorotrifluoroethane) was added and the sample mixed to separate lipids and yolk-sacs in the oocyte from proteins. Nuclei were simply mixed using a vortexer to disrupt the nuclear membranes and homogenise.

Oocytes were then centrifuged at 13,000r.p.m. for 1 minute and then the supernatant was transferred to a fresh tube. A quantity of SDS-PAGE sample buffer equal to the nucleus/cytoplasm sample volume was added to the sample before loading half of this volume (unless stated) onto the acrylamide gel for SDS-PAGE separation.

Preparation of Poly(A) structures from oocytes

Affinity purification was used to prepare polyadenylated (polyA) structures from oocytes. From the polyA structures, packaged maternal mRNA, mRNP protein components and nascent polypeptides emerging from polysomes (polyribosomes) can be extracted.

Oocytes (stage I-III) were collected following dissection from ovary and experimental treatment and homogenised in a volume of binding buffer of 1.5-2.5µl per oocyte. A slightly smaller volume of freon was then added and then mixed to extract lipids from the oocytes followed by centrifugation for 1 minute at 13,000rpm and retention of the supernatant. Oligo-d(T) cellulose affinity beads (Amersham Pharmacia) were then prepared to bind poly(A) structures by washing twice in water and then once in binding buffer. A $\frac{1}{10}$ th volume of oligo-d(T) resin suspension was added to each oocyte sample. The resin and sample mixture was subsequently incubated on ice for 1 hour to allow binding to occur followed by centrifugation at 13000rpm for several seconds and discarding of the supernatant containing non-polyA structures — the poly(A)-fraction.

Four times the original homogenised sample volume of binding buffer was then used to resuspend the beads by mixing and the beads centrifuged for several seconds at 13000rpm before discarding the supernatant. This

washing was repeated in total three times. Finally to prepare the poly(A)+ fraction for SDS-PAGE, a $\frac{1}{10}$ th of the original homogenised sample volume of SDS-PAGE sample buffer was added to the beads and the suspension boiled for 2 minutes.

Oligo-d(T) binding buffer

0.2M NaCl

20mM Tris-HCl (pH 7.6)

2mM MgCl₂ (disrupts ribosome structure)

Add 1mM DTT (inhibits oxidation of proteins) before use.

Trichloroacetic acid (TCA) precipitation of radiolabelled oocyte proteins

Oocytes were squashed with a glass pipette onto filter paper before addition of 5% TCA and incubation on ice for 30 minutes to precipitate proteins onto the paper surface. Free radiolabel including tRNAs charged with the label were removed by washing with TCA three times. The filter papers were then washed in 95% ethanol and dried before incubation with a solution of 100µg/ml ribonuclease A for 10 minutes. Finally the filters were washed again in 95% ethanol, dried and then radioactivity counted using a Geiger counter.

SDS-PAGE, Coomassie staining, electroblotting and immunodetection

SDS-PAGE

Separation of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) used the gel composition and running buffer detailed below. In general, protein samples were mixed with an approximately equal volume of SDS-PAGE sample buffer prior to loading onto the polyacrylamide and boiled for two minutes. Proteins were loaded in amounts of 15-25 μ l alongside approximately 10-15 μ l of briefly heated molecular size protein markers.

Gel

Reagent	<u>12% running gel</u>	<u>Stacking gel</u>
40% Acrylamide soln	1.2ml	200 μ l
3M Tris-SO ₄ ²⁻ (pH8.3)	0.5ml	—
0.5M Tris-SO ₄ ²⁻ (pH6.9)	—	156 μ l
dH ₂ O	1.63ml	870 μ l
Glycerol	0.6ml	—
10% SDS	60 μ l	18 μ l
25%		
Ammonium Persulphate	5-7 μ l	2-4 μ l
TEMED	7 μ l	4 μ l

Running Buffer

Tris	3 g/l
Glycine	14.4 g/l
10ml	10% SDS

Gels were run at 200V for approximately 1½ hours.

Coomassie Staining

When subsequent immunoblotting of SDS-PAGE gels was not a

requirement, separated proteins were visualised by immersion in coomassie blue for 1 hour with gentle agitation. In order to remove background stain from the gel and reveal stained protein material more clearly, the gel was subsequently de-stained, changing the de-staining solution at intervals.

Coomassie brilliant blue stain

Coomassie brilliant blue	1g
90% ethanol	250ml
Glacial acetic acid	50ml
dH ₂ O	200ml
(Total 500ml)	

Coomassie destain

90% ethanol	250ml
Glacial acetic acid	100ml
dH ₂ O	650ml
(Total volume 1000ml)	

Electroblotting

In order to detect specific proteins separated by SDS-PAGE by antibodies, proteins were first transferred to a nitrocellulose membrane by use of electric charge (electroblotting).

Firstly, the polyacrylamide gel was washed in transfer buffer, the composition of which is given below. Also, 4 pieces of 3M filter paper and the nitrocellulose membrane were soaked in transfer buffer.

Transfer buffer

Tris 3g

Glycine 11g

Methanol 200ml (20% v/v)

Tris and Glycine dissolved in 800ml water then made up to 1l with methanol

The electroblotting was performed in a semi-dry electrophoresis tank with the components of the transfer in the following order relative to the anode: 2 filter-papers, nitrocellulose membrane, SDS-PAGE gel, 2 filter-papers. A current of 36milliamps was then set for the transfer to proceed over approximately 2 hours.

Immunodetection

Membranes carrying blotted proteins were treated as follows in order to reveal positions of specific proteins. Reagents are given below. The membrane was immersed overnight in 10% powdered skimmed milk in phosphate-buffered saline (PBS) in order to block unbound sites on the nitrocellulose.

The milk was then discarded and the filter rinsed in PBST (PBS and the detergent Tween) and immersed for 10 minutes with rocking of the container. The membrane was then incubated with a solution of PBST containing the appropriate dilution of the primary antibody and incubated for an hour at room temperature with rocking. After this time the antibody was discarded and the membrane rinsed four times in PBST during a time

period of 30 minutes.

Next, a solution containing the appropriate dilution of the secondary antibody was added to the membrane for incubation at room temperature for 1 hour, again gently agitating the membrane with rocking. The secondary antibody was then discarded and the membrane washed again four times in PBST during 30 minutes.

Finally, the position of the bound antibodies was then revealed using the ECL reaction (Amersham Pharmacia) which generates light to expose photographic film by reacting with the horseradish peroxidase-conjugated secondary antibody. Briefly the membrane was soaked in a solution containing 0.5ml reagent A and 0.5ml reagent B. Then the membrane was positioned within plastic cling-film in an exposure box. In the dark a piece of photographic film was cut to shape and positioned on the membrane and sealed in the exposure case for a minimum of 2 minutes.

Lastly, the film was removed and washed in developer and then fixative with draining and rinsing in water between washes. Regions of peroxidase activity and thus, bound antibodies, on the original membrane would be reflected by the relative positions on the exposed film.

Reagents for immunodetection

2 x [1 PBS tablet / 200ml H₂O giving solution of pH7.4 and 0.01M phosphate]

10% powdered skimmed milk (20g/ 200ml PBS)

50ml PBS - keep

PBST: 150ml PBS- add 100µl Tween20

1° antibody: in general in a 1:10,000 dilution

2° antibody: horseradish peroxidase-conjugated antibody raised against primary and binding to the constant region of the antibody's structure; in general in a 1:5,000 dilution

Stripping of nitrocellulose membranes

In order to repeat the immunodetection protocol to detect the presence of another protein with a different primary antibody using a nitrocellulose membrane previously treated, the membrane was stripped of the antibodies currently bound. This involved incubating the membrane in stripping solution at 50°C for 30 minutes within a hybridisation oven. Following the incubation the stripping solution was poured off and the membrane blocked again before treatment with relevant antibodies as described above.

Stripping solution

SDS 2%

2-mercaptoethanol 100mM

Tris-HCL (pH6.7)

Volume made to 50ml with water

Preparation of Immunoglobulin G molecules from serum

IgG molecules were prepared by ion-exchange purification from 250µl volumes of serum each time. An approximate $\frac{1}{10}$ th volume of protein A beads (Amersham Pharmacia) was first washed through mixing, allowing the beads to settle and discarding supernatant several times in approximately 1ml of PBS. Then 500µl of PBS was added to the beads and 250µl of serum was added to the suspension before mixing on a rotary mixer for 1 hour and adding 250µl more of PBS during this period. At the end of the mixing period, the beads were allowed to settle and the supernatant discarded before the beads were washed several times with PBS. After removing the PBS the beads were washed again in distilled water. Approximately 2x volume of elution buffer was then added to the settled beads and the suspension mixed several times before transferring the supernatant to a fresh tube. Then a $\frac{1}{10}$ th volume of Tris-HCl (pH8) was added to neutralise the elution volume.

Elution buffer

0.1M Glycine

Adjust to pH 3.0 with HCl

Immunoprecipitation of mRNP

Complexes of mRNP were extracted by precipitation from oocyte extracts using antibodies. Oocyte samples were first prepared using freon as described above after addition of a volume of approximately 5 μ l, unless stated, of mRNP binding buffer per oocyte for homogenisation. Then approximately 1.5ml of borate buffer was prepared (borate being used in preference to Tris whose amine groups would saturate the affinity beads used subsequently, see below). NaCl was then added to a concentration of 0.15M.

10 μ l of Prosep-A affinity beads (Prosep) were washed several times in 100 μ l of the above borate/NaCl solution then a volume of the solution removed to leave a volume of approximately 50 μ l. The beads were present to immobilise the antibody upon its subsequent addition in a volume of 2.5 μ l. The bead-containing solution was then rotated to mix at room temperature for 1 hour. The supernatant was discarded and binding buffer added to give a total volume of approximately 100 μ l. Dimethyl pimelimidate was added to the solution to a concentration of 20mM in order to crosslink the antibodies permanently to the beads and the solution mixed for half an hour. Next, 1ml of 0.2M of ethanolamine was added to saturate the remaining binding surfaces of the beads and the beads then washed twice with PBS discarding most of the supernatant at the end of

the last wash to leave a solution of mainly antibody-complexed beads. Approximately 5 μ l of the antibody-bead complexes were added to each oocyte sample and the samples mixed for half an hour. The beads were washed twice with mRNP binding buffer, the buffer removed and 20 μ l of Laemmli buffer added to the beads before boiling for 2 minutes. Half of this volume was then loaded onto SDS-PAGE gels.

Borate buffer: 0.1M Sodium Borate, pH8.5

Binding buffer:

50mM NaCl

20mM Tris (pH7.6)

1mM MgCl₂

Photocrosslinking of mRNP complexes

To test for proximity of mRNP proteins, oocyte extracts were photocrosslinked with UV-light. Samples were transferred to the wells of a glass dish coated previously with Sigmacote (Sigma) to reduce adhesion and exposed to UV-light at 254nm for 20 minutes. The glass dish was kept on ice for this duration before collecting the irradiated samples into tubes and placing on ice.

Agarose Gel Electrophoresis

Agarose gels were made to 1% by dissolving 0.5g agarose at 100°C in 50ml Tris acetate EDTA (TAE) buffer. Ethidium bromide was added during the cooling of the molten agarose to a concentration of 0.5µg / ml. After casting and setting, the gel was covered in TAE buffer in the electrophoresis tank. Higher agarose concentrations were obtained by increasing the agarose quantity dissolved accordingly.

DNA or RNA samples were loaded onto the gel with $1/10$ th total volume loading buffer (see below). The gel was run at 80v for approximately 1-1½ hours and visualised with a UV-light transilluminator. Images were recorded and processed with the GeneSnap (Synoptics Ltd) software package.

Intensities of recognised bands were calculated by the GeneTools (Synoptics Ltd) densitometry software package.

Tris-acetate EDTA buffer (TAE)

50x stock (litre)

Tris base 242g

Glacial acetic acid 57.1ml

0.5M EDTA (pH 8) 100ml

1x running buffer

Tris-acetate 0.04 M

EDTA 0.001 M

(*i.e.* 50x diluted stock)

TAE Loading buffer

Glycerol 50% v/v

Bromophenol blue 0.42% w/v

Xylene cyanol FF 0.42% w/v

Make up to desired volume with H₂O

Restriction enzyme digestion of DNA

Digestions in general were undertaken in a total volume of 20 μ l using 2 μ l enzyme for a single digestion or 1 μ l of each enzyme for a double-digestion and 2 μ l of the appropriate buffer ($1/_{10}$ th total volume). The amount of DNA added varied and water was added to bring the final volume to 20 μ l.

Commonly for example 5 μ l of DNA was used, together with 11 μ l water.

If desired, 2 μ l of 1000 U/ml calf intestinal alkaline phosphatase (Amersham Pharmacia) was added during the last 30 minutes of the reaction in order to remove terminal phosphate groups from digested nucleic acid strands to prevent annealing of the single-strand overhangs.

The digestion reaction was then terminated if ligation was a subsequent step by heat-treating at 65°C for $1/2$ hour to inactivate nucleases. After the heat treatment, 1 μ l 1:100 Ribonuclease A (Sigma) was added and left to digest any RNA in the mixture for 15 minutes at room temperature.

Ligation of DNA

DNA fragments digested with restriction enzymes were ligated using 'Ready-to-go' ligation tubes (Amersham Pharmacia).

Ready-to-go ligation

The dried ligation mix was first hydrated by the addition of 10 μ l of DEPC-treated water. A 2:1 ratio of volumes of vector and insert DNA was then added to the tube before making the final volume up to 20 μ l. The ligation reaction was then left to proceed for approximately 40 minutes at room temperature before diluting with 80 μ l of DEPC (diethyl pyrocarbonate)-treated water for storage at -20°C after inactivating the ligation reaction by treating at 70°C for 10 minutes.

Reaction mix

Reaction mix made up to 20 μ l with water contains:

66mM Tris-HCl (pH7.6)

6.6mM MgCl₂

0.1mM ATP

0.1mM spermidine

10mM DTT

DNA extraction from agarose gels

In order to extract DNA which had resolved as a clear distinct band on an agarose gel after electrophoresis the band was cut out with a sharp blade as close to the fluorescing ethidium bromide-stained area as possible. The

agarose sample was then transferred to an eppendorf tube and approximately 3 volumes of 3M sodium iodide was added until the agarose had dissolved.

Then, 20 μ l of glass-milk (silica resin solution, see below) was added and the mixture left for 20 minutes. The mixture was then washed three times with GeneClean™ washbuffer (Promega) by adding 0.5ml each time followed by spinning at 13,000rpm for 30 seconds and removal of supernatant. The resin was then left to dry and then, in order to subsequently elute bound DNA, 40 μ l of H₂O was added and left to incubate at 50-55°C for 5 minutes.

Finally the mixture was spun for 30 seconds and the supernatant collected for storage at -20°C.

Glass milk

NaI	3M
Silica resin	100mg/ml

Wash buffer

NaCl	50mM
Tris-HCL (pH 7.5)	10mM
EDTA	2.5mM
Ethanol	50% v/v

Plasmid mini-prep

Plasmids were extracted from 10ml of overnight bacterial culture growing in LB selective medium and applying the Wizard® Plus Mini-prep.

purification method (Promega). One Wizard minicolumn was prepared per miniprep. by removing and keeping the plunger from a 3ml disposable syringe then attaching the syringe barrel to a Wizard mini-column. Then 1ml of resuspended resin (DNA purification resin) was pipetted into the barrel.

Lysate from the overnight bacterial culture was prepared by centrifuging at 1,400g for 10 minutes then decanting the supernatant. The pellet was then resuspended in 400 μ l cell resuspension solution and transferred to a 1.5ml microfuge tube. 400 μ l of cell lysis solution was added to the resuspended cells and mixed by inverting. Then 400 μ l of neutralisation solution was added and the lysate mixed. The lysate was then centrifuged at 10,000g briefly or for several minutes until a pellet was visible. Finally, the lysate was transferred to the barrel of the Wizard column/syringe assembly and pushed through by re-inserting the syringe plunger. A volume of 2ml of column wash solution was then washed through the column/syringe barrel assembly after removing and then reattaching the plunger. The Wizard column was then transferred to a 1.5ml microfuge tube and spun at 10,000g for 2 minutes to remove excess liquid from the resin of the column. The column was subsequently transferred to a fresh tube and 50 μ l of nuclease-free water added and left for 1 minute before centrifuging for 20 seconds at 10,000g to elute plasmid DNA from the

resin. Plasmid DNA was stored in water at -20°C.

Cell resuspension solution

50mM Tris pH7.5

10mM EDTA

100µg/ml ribonuclease A

Cell lysis solution

0.2M NaOH and 1% SDS

Neutralisation solution

1.32M potassium acetate

Column wash solution

80mM potassium acetate

8.3mM Tris-HCl pH7.5

40µM EDTA

55%w/v 95% ethanol

Transformation by heat shock

Plasmid transformations were performed using *NovaBlue E.Coli* cells from Promega.

A volume of 1-2µl of the DNA to be introduced into the cell was added to the 50µl cell suspension provided and the cells subsequently kept on ice for 5 minutes. The cells were then transferred to a water bath with a temperature of 42°C for 30 seconds and then moved immediately back to ice as per manufacturer's instructions. Upon transfer 250µl of provided SOC medium was used to dilute the cell suspension and 50µl of medium

was spread onto an ampicillin-resistance selective plate to select for those cells transformed with ampicillin-resistance-conferring vectors. Growth of cells was checked for after incubation of plates overnight at 37°C.

RNA purification from oocytes by phenol-chloroform extraction

Oocyte nuclear or cytoplasmic samples were homogenised by mixing with 3-4µl of extraction buffer per nucleus or cytoplasm. An equal volume of freon was then added to the cytoplasm, vortexed and centrifuged at 13,000rpm for 1 minute before retaining the upper phase.

Then $\frac{1}{10}$ th volume proteinase K in 10% SDS solution was added to the homogenised nuclei/freon-extracted cytoplasm and left at room temperature for 30 minutes. An equal volume of phenol-chloroform was subsequently added to homogenised nuclei/freon-extracted cytoplasm before centrifugation at 13,000rpm for 1 minute. The upper phase was then transferred to a fresh tube and the extraction procedure repeated with chloroform alone. The upper phase was transferred again to a fresh tube and then $\frac{1}{10}$ volume 3M Sodium acetate was added together with 3 volumes of 100% ethanol. The solution was then left at -80°C for approximately 2 hours to precipitate nucleic acids.

Solutions were then centrifuged at 10,000 r.p.m. for 40 minutes at 2°C and

the supernatants decanted after which the remaining pellets were dried. Finally the pellets were resuspended in DEPC (diethyl pyrocarbonate)-treated water of volume equivalent to approximately 1µl per nucleus or cytoplasm unless stated. RNA solutions were divided into aliquots if necessary and stored at -70°C .

RNA Extraction buffer

0.5M sodium chloride
20mM Tris-HCl pH7.2
5mM EDTA

Phenol-chloroform

50:50 mixture of phenol pH 4.5 and chloroform

Proteinase K solution

1mg / ml proteinase K in 10% SDS solution

Polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR)

Amplification of nucleic acids was done using the 'one-step' protocol, as provided with commercial RT-PCR and PCR beads (Amersham Pharmacia). For reverse-transcription of purified mRNA, RT-PCR primers (appropriately designed to flank the coding region of the target gene and including required restriction sites if required) were diluted as stated by suppliers with DEPC-treated water to give a concentration of 10µM. The dilution procedure applied to PCR primers as well.

The beads provided for a single RT-PCR reaction were dissolved in 46µl of DEPC-treated water (or greater to make the final volume to 50µl), keeping the reaction mixture on ice during the preparation. Then, 0.5-2µl (unless stated) of template DNA or RNA was added to the reaction mixture followed by 1µl (unless stated) of each primer. The final reaction volume in PCR tubes was 25µl and the appropriate volume of DEPC-treated water was added accordingly. The reaction tube was then placed in the thermal cycler and the appropriate programme started.

The details of the cycling programmes used for PCR and RT-PCR are given below. Standard PCR involved cycles of denaturation at 95°C, annealing at 40-60°C and elongation at 72°C. For RT-PCR, the initial temperature was 42°C to allow reverse transcription to proceed. This was followed by a denaturation of 95°C for 5 minutes to denature template DNA and inactivate the reverse transcriptase.

Annealing temperatures were adjusted according to the homology of primers to the template DNA.

PCR

34 cycles of:

<u>°C</u>	<u>minutes:seconds</u>
94	1:0
53	0:45
72	1:25

Final extension stage of 72°C for 10 minutes followed by holding temperature at 0°C.

RT-PCR

<u>°C</u>	<u>minutes:seconds</u>
42	25:0
95	5:0
Followed by 30 cycles of:	
95	1:0
47	0:45
72	1:30

Final stage of holding temperature at 0°C.

Construction of expression vectors

T7-*Xp54* expression vector

In order to construct the T7-tagged p54 plasmid expression vector a PCR cDNA fragment containing the coding sequence of the *Xp54* gene was obtained from a bacterial clone expressing the Xp54 protein (Ladomery *et al.*, 1997) and inserted by ligation between the unique *Xba*I and *Bam*H1 sites of the pCGT7 vector (Smillie and Sommerville, 2002). The pCGT7 is a modified version of the pCG vector as shown in figure 13(b). The vector was maintained and propagated in Novablue cells.

A variant of the *Xp54* coding sequence mutated to cause the amino acid substitution of the glutamic acid (E) residue of the DEAD motif to glutamine (Q) was similarly inserted in the pCGT7 vector to construct the T7-tagged p54 DQAD mutant. Mutagenic primers were used to amplify by PCR two halves of the *Xp54* coding sequence from the Xp54-expressing bacterial clone. The two DNA PCR reaction products were joined in a

third PCR reaction to generate a fragment containing a reading frame for the translation of the whole *Xp54* protein with a mutated DEAD-box encoding region.

All PCR fragments generated for inclusion in expression vectors were visualised on 2% agarose gels and extracted as described above.

The result of the ligation is the *Xp54* coding sequence fused downstream of a T7-gene tag, of use as an epitope in immunological applications. The resulting protein structural organisation is represented in figure 13(a). Ligated solutions were then used for transformation of bacterial hosts as described above for subsequent large scale recovery of the plasmid.

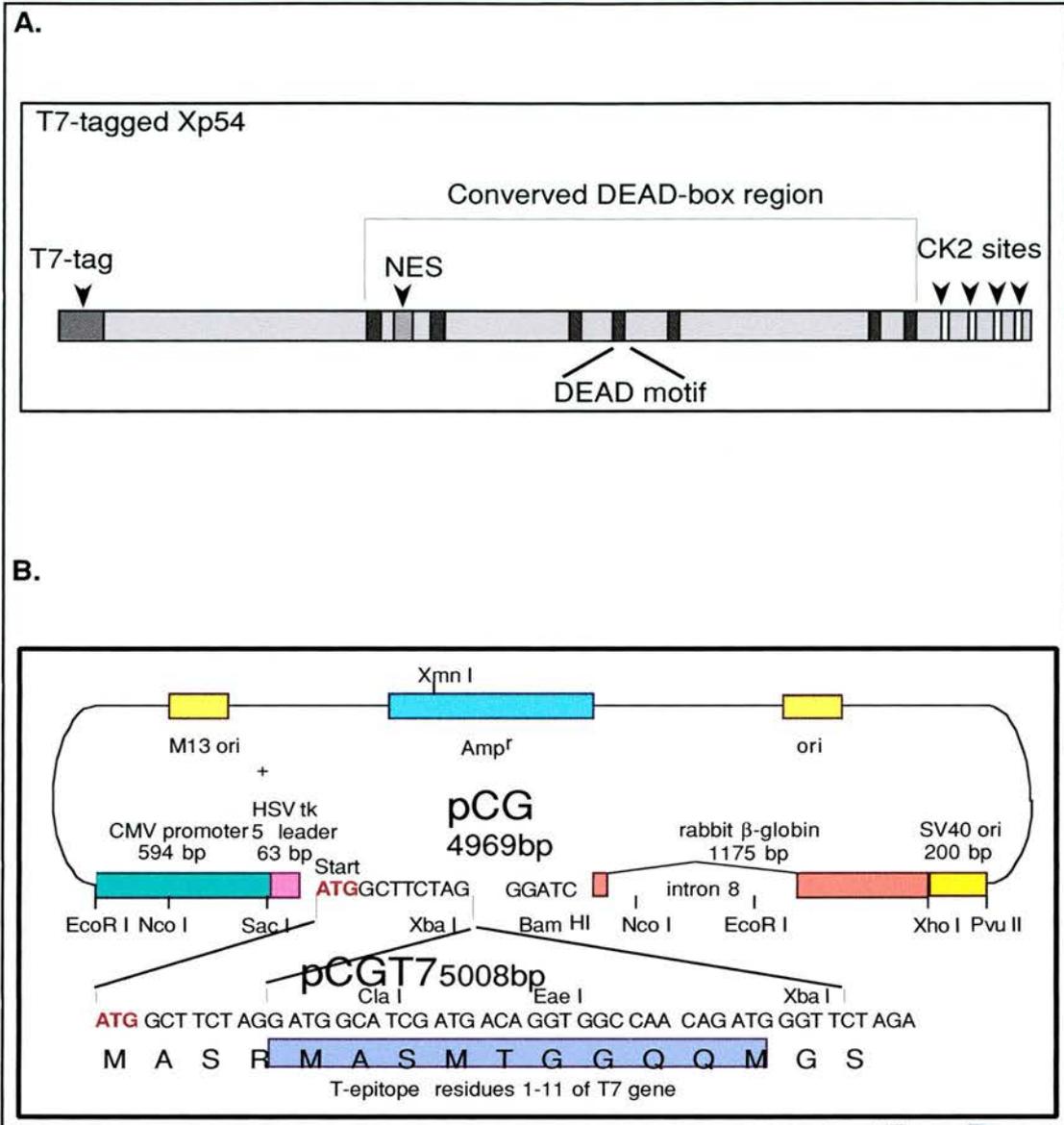


Figure 13: Construction of T7-tagged Xp54 expression vector.

(a) Recombinant T7-tagged p54

Dark-grey boxes represent conserved regions found in DEAD-box RNA helicases. The nuclear export sequence (NES) and the casein kinase II phosphorylation sites described in Introduction are shown. Light-grey areas are other regions of the Xp54 enzyme.

(b) Expression vector pCGT7

pCG contains a CMV promoter enabling transcription in eukaryotic cells such as the *Xenopus* oocyte and a leader sequence to initiate translation. In the pCGT7 derivative (lower sequence) of pCG a fragment of the viral T7-gene encoding the T-epitope is located immediately upstream of a unique *Xba*I restriction site. The rabbit β -globin 3'UTR is located immediately downstream of a *Bam*HI site. Various other origins of replication are shown of use in alternative systems along with other restriction enzyme sites.

DNA sequencing and protein sequence analysis and alignment

Cloned *Xenopus* cDNAs were identified through the expressed sequence tag (EST) databanks of the European Bioinformatics Institute (www.ebi.ac.uk). The relevant IMAGE clones were ordered from the MRC Geneservice (Cambridge, UK; www.hgmp.mrc.ac.uk/geneservice/index.shtml). Sequencing was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland (www.dnaseq.co.uk) using an Applied Biosystems model 3730 automated capillary DNA sequencer. The method uses fluorescent dye-conjugated di-deoxynucleotide terminators corresponding to each of the four DNA bases. Fragments are produced after multiple rounds of primer annealing and extension by Taq DNA polymerase then strand separation ending in the dye-labelled base corresponding to the nucleotide base of the template. The thermocycling reaction is carried out in a single tube and the fragments separated by electrophoresis on a polyacrylamide gel with the four types of fluorescence being detected after excitation with laser light. Sequencing primers for thermocycler sequencing were designed to be approximately 20 nucleotides in length (20-mer oligonucleotides) with approximately 50-55% GC content and a melting temperature (T_m) ideally of less than 65°C:

$$T_m = ((A+T) \times 2) + ((G+C) \times 4)$$

Although only one primer is required for thermocycler sequencing, separate primers were designed to sequence upstream or downstream of an EST to obtain unsequenced stretches as necessary. Primers were submitted at a concentration of 3.2 μ M.

Template (as double-stranded, plasmid DNA) was extracted from an overnight culture of the IMAGE clone bacterial host using the Mini-prep method described above. It was then submitted for sequencing at a concentration of 20 μ g/ml to obtain in the 15 μ l used in a sequencing reaction a quantity of around 300ng. Plasmid concentration was quantitated against Lambda DNA of 100 μ g/ml purchased from Promega. Protein sequences, molecular weight, amino acid composition and charge distribution were produced from sequenced cDNAs as required using the Statistical Analysis of Protein Structure (SAPS) online tool (accessible from ExPASy: <http://ca.expasy.org/tools> or the EBI: www.ebi.ac.uk/saps). Protein sequence alignments were undertaken using the online EMBOSS-align sequence alignment tool at the EBI website. This employs the Needleman-Wunsch global alignment algorithm which tests every possible alignment of two protein sequences and produces a scoring matrix containing values for all residue matches. The alignment with the greatest total score is taken to be the optimum. For each gap inserted in a sequence by the algorithm to enable a particular alignment a penalty is subtracted

from the score as is a penalty for the total number of gaps albeit less severe. These are referred to as the gap open penalty and gap extension penalty respectively.

The optimal alignment is produced by the algorithm alongside the percentage identity and percentage similarity between the two sequences. Percentage identity refers to the number of identical matches between the two sequences expressed as a percentage of the total length in nucleotides of the aligned region. This total starts from the first nucleotide whether matched/mis-matched or opposite a gap to the last nucleotide matched/mis-matched or opposite a gap.

The percentage similarity refers to the percentage of nucleotides over the same aligned region which have a scoring matrix value greater than or equal to 0.

Results

The steady-state levels of different mRNAs change throughout the course of oogenesis and have different stabilities

The proposed investigation into Xp54's influence on mRNA export and stability in the oocyte is to involve bringing about perturbations in cellular Xp54 levels via overexpression of the protein or reducing its expression. Thus any interpretation of results arising from these experiments would be expected to benefit from an examination of the relative levels of a range of mRNAs under steady-state conditions, with which Xp54 is understood to associate in mRNP particles or with which Xp54 may potentially associate. To this end oocytes were isolated from dissected *Xenopus* ovary representing all 6 Dumont stages (Dumont, 1972). Stage 1 and 2 oocytes were collected together into a volume estimated at 1000 oocytes. Subsequently stage 3-6 oocytes were collected in numbers that approximately equalled the total volume of the stage 1-2 oocyte sample. This equated to : 200 stage 3 oocytes; 100 stage 4 oocytes; 50 stage 5 oocytes; 40 stage 6 oocytes.

Oocytes were then homogenised with the same volume (400µl) of RNA extraction buffer and RNA extracted as described in Methods and Materials, the final RNA pellets being resuspended in a total of 100µl of water. Volumes of 2µl of RNA were then used as a template for a series of RT-PCR reactions that targetted a set of specific mRNAs in order to gain a readily detectable reflection of the levels of each mRNA in each Dumont-stage sample as judged by the relative intensities of the final, amplified cDNA products. The mRNAs amplified were chosen to represent those that are understood to be translated before and/or after maturation induction or those that are not translated until fertilisation of the oocyte. The amplified mRNAs believed to be translated before oocyte maturation were *ribosomal proteins L1* and *S1* (*rpL1* and *rpS1*) and *histone H4*. Messages for *mos*, *cyclin B1* and *histone B4* were chosen to represent those genes only translated after maturation induction. *FRGY1* was chosen to represent an mRNA that is translated only after fertilisation. Densitometry was subsequently performed on the mRNA bands for the oocyte-stage samples for each gene in order to obtain a figure representing the relative band intensities that could be plotted graphically. Both the intensity for the total oocyte mRNA sample and the intensity equivalent per single oocyte were

calculated and plotted. The results are shown in figure 14(a) and 14(b).

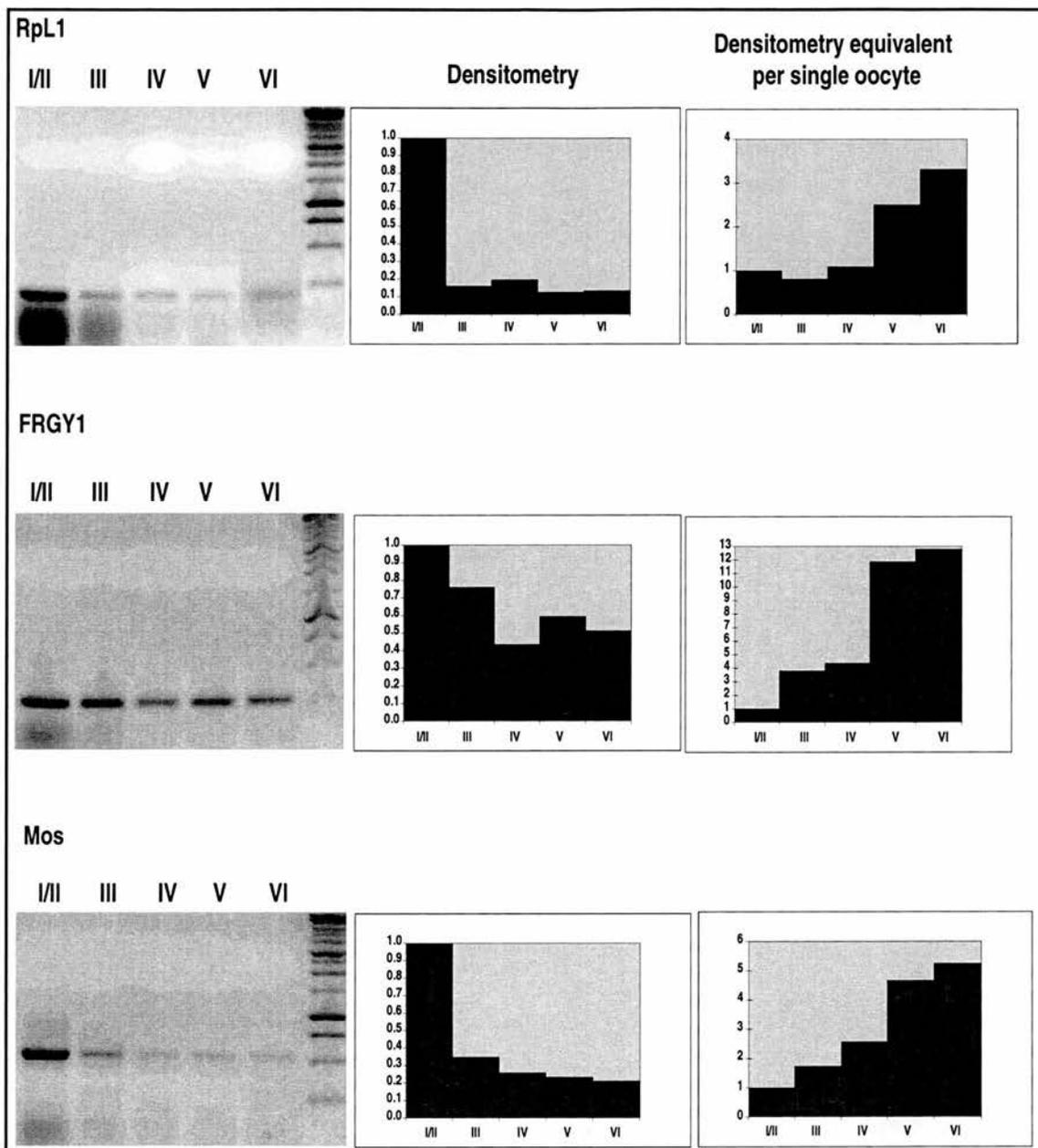


Figure 14: Oocytes accumulate mRNAs of a wide range of types through oogenesis. RT-PCR reactions were performed for a range of mRNAs using RNA extracted from sets of oocytes at different stages (I-VI) of oogenesis of approximately equal volume. Densitometry was performed on the cDNA gel bands for each sample using GeneTools™ software (Synoptics Ltd). The resulting figures are presented normalized to the first sample (I/II) given a value of 1 and presented as an equivalent value for a single oocyte, again normalized to the first sample. Equivalent values were calculated by dividing by the estimated (stages I/II) or counted number (stages III-VI) of oocytes for that sample (see text).

(a) Agarose gels and densitometry graphs showing RT-PCR reaction products (cDNA) for the genes *rpL1*, *FRGY1* and *mos*. Size markers are shown on the right-hand side of the gel and the four lowest represent 1,000, 750, 500 and 250bp.

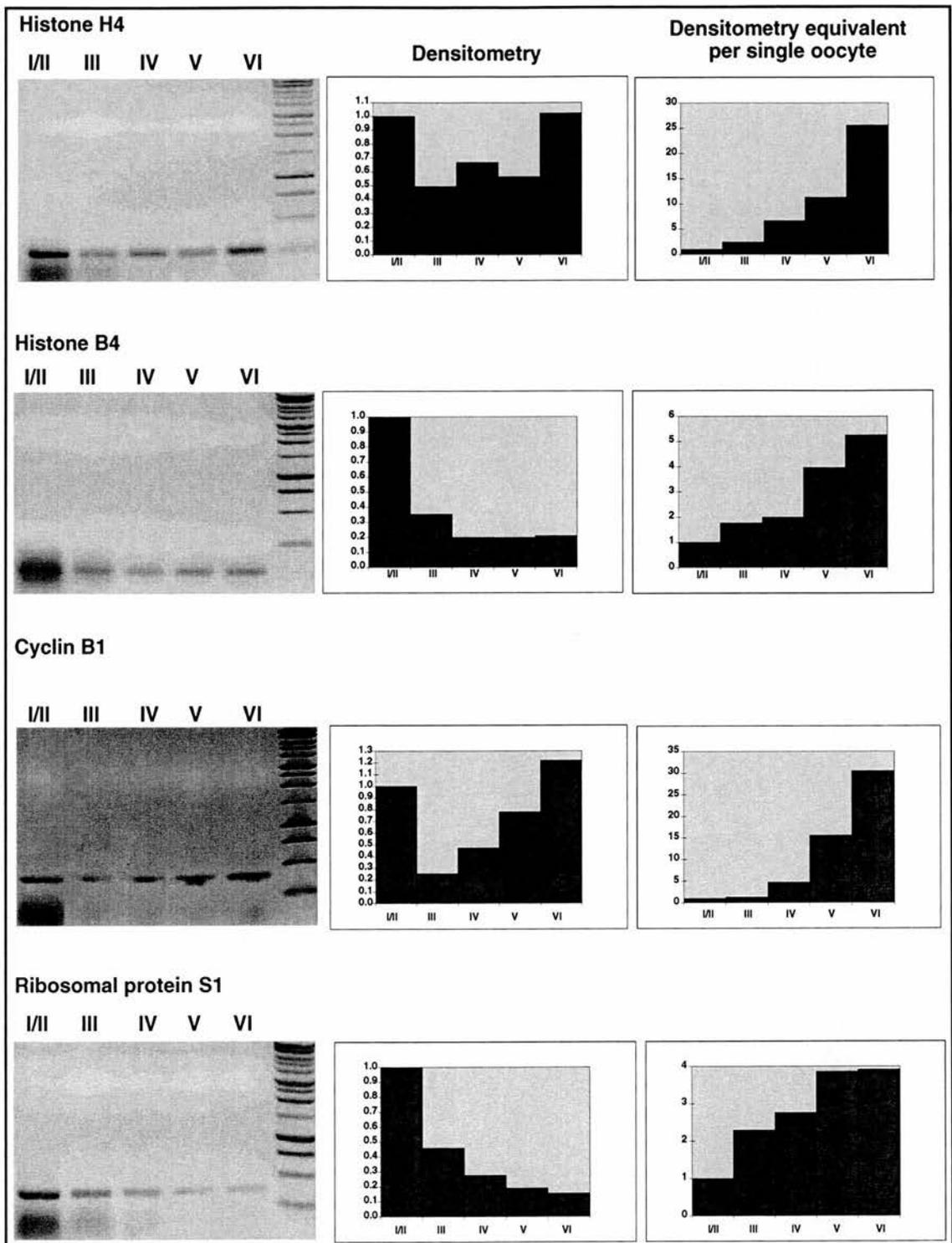


Figure 14(b): Oocytes accumulate mRNAs of a wide range of types through oogenesis.

Details as in fig.14(a) except agarose gels and densitometry graphs show RT-PCR reaction products (cDNA) for the genes *histone H4*, *histone B4*, *cyclin B1* and *rpL1*. Size markers are shown on the right-hand side of the gel and the four lowest represent 1,000, 750, 500 and 250bp.

The mRNA levels per mass of oocytes show different patterns of change for different genes during oogenesis and the total amounts of different mRNAs present at a given stage of oogenesis vary as can be seen in the cDNA bands of the separate agarose gels in figure 14. For example, *FRGY1* shows particularly high levels of mRNA per mass of oocyte at an early stage relative to *histone H4* but as oogenesis proceeds this level does not increase further in contrast to increases in total mRNA seen in *histone H4* later in oogenesis. However, it is noticeable that most for most of the mRNAs analysed here, there is a gradual or consistent accumulation in the oocyte during oogenesis. This applies to classes of genes with different translational status such as *rpLI* which is translated before oocyte maturation and *cyclin B1* or *mos* which are not translated until maturation induction yet which continue to accumulate their mRNA. In the example of *FRGY1* and *histone H4* the different patterns of mRNA change reflected in the agarose gel analyses both translate into consistent increases when expressed per single oocyte.

Thus it appears that a one-directional change rather than fluctuating levels of mRNA in the oocyte is the norm for mRNAs of all classes of translational status during oogenesis.

However, the means by which the mRNAs of different translational status

are accumulated may differ in that those mRNAs that are not needed until maturation induction or after fertilisation — yet are apparently being transcribed such as *histone B4* and *mos* (fig.14) — are presumably stored as mRNP, potentially containing Xp54. Thus by inhibiting transcription and using the RT-PCR method to examine levels of specific mRNAs at intervals, information on the stability or half-life of the mRNA should be visible as should an indication of whether an mRNA persists in the absence of transcription. Thus stage 4 oocytes were incubated at room temperature in buffer containing 0.5µg/ml of the transcription inhibitor alpha-amanitin or incubated without the inhibitor. 15 oocytes were sampled at 0, 6, 12, 24, 36 and 48 hours from the alpha-amanitin treated oocyte pool and at 48 hours from the control pool. RNA was then extracted from the oocytes as described in Methods and Materials and, as above, volumes of 2µl of RNA were then used as a template for RT-PCR reactions that targeted a range of specific mRNAs understood to have different translational status in order to gain a reflection of the levels of each mRNA in each of the 7 samples. The corresponding gels are shown in figure 15.

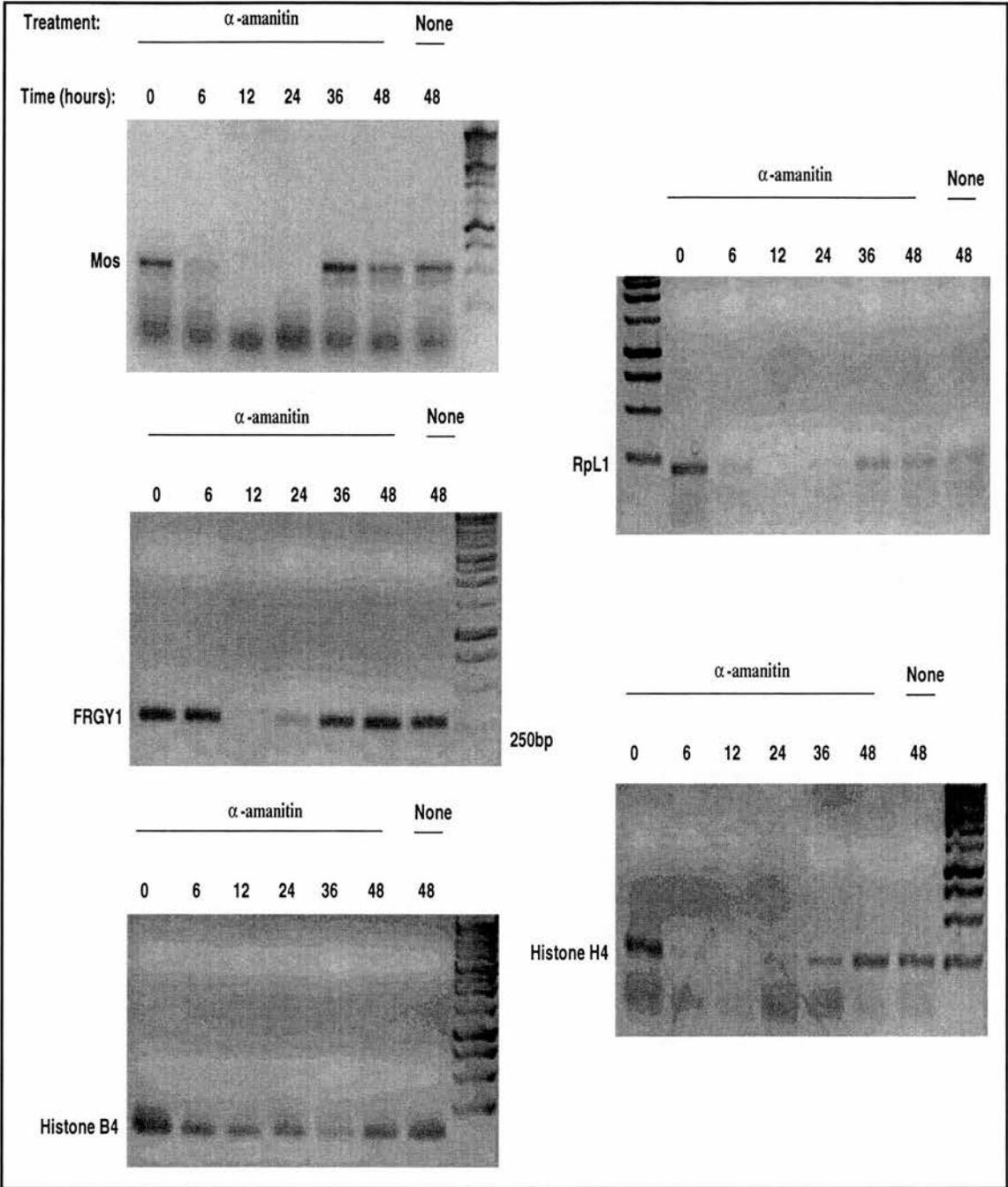


Figure 15: Stabilities of oocyte mRNAs differ according to the gene of origin.

RNA was extracted from stage 4 oocytes treated or not treated with 0.5 μ g/ml α -amanitin at intervals and used for RT-PCR reactions that targeted the mRNAs of specific genes for amplification. Agarose gels are shown for these genes showing a reflection of the relative amounts of the mRNA at each time interval. The lowest four marker sizes are 1000bp, 750bp, 500bp and 250bp (indicated for *FRGY1* gel).

A variable effect of alpha-amanitin on mRNA stabilities is seen in fig.15 according to the gene of origin in terms of the rapidity and effectiveness of action. For *rpL1*, *histone H4*, *mos* and *FRGY1* mRNA, the treatment appears to abolish transcription completely with the result that remaining mRNA from that particular gene quickly degrades and 12 hours following treatment, no transcripts are detectable in the oocyte. This mRNA degradation alternatively may represent an indirect effect of the inhibition of expression of another gene which results in transcription of *rpL1*, *histone H4*, *mos* and *FRGY1* being inhibited also. *Mos* mRNA and *rpL1* mRNA appear to show the slowest recovery with *histone H4* and *FRGY1* showing higher levels at 24 hours post-treatment. *Histone B4* mRNA remains detectable following treatment of the oocytes with alpha-amanitin although a decline is noticeable, particularly at 36 hours. The levels do not seem to recover to those of the time 0 sample although comparison of the 48 hours sample with the non-treated sample indicates that recovery from any disruption to mRNA stability brought about by alpha-amanitin has occurred.

Thus differences clearly exist between genes in the total stability of their mRNAs with some genes apparently being dependent on ongoing

transcription for their continued presence and one apparently having a residual storage.

mRNAs differ in the degree of their association with Xp54-containing structures

A selection of those mRNAs analysed in their accumulation or stability were investigated to confirm suspected association with Xp54 in the oocyte. Stage 4 oocytes were injected with the T7-*Xp54* vector and incubated at room temperature for 48 hours, followed by extraction of polyadenylate structures and immunoprecipitation of T7-containing structures from the resulting material. In this way, it was hoped to obtain mRNAs in the same structures that T7-*Xp54* had associated with following microinjection of the expression vector and thus gain a rough idea of what diversity of mRNA probably interacts with Xp54 during oogenesis.

Antibodies raised against Xp54 in previous experiments have not precipitated such complexes and thus anti-T7 antibodies were used instead (J.Sommerville, pers. comm.).

Poly(A)+ structures were extracted as described in Methods and Materials except 35 oocytes were collected and homogenised in 200µl of oligo-d(T) extraction buffer before freon extraction. Also, elution from the affinity

beads was carried out, after washing of beads as described, with three separate additions of water at 50°C and removal after 3 minutes incubation. The pooled 150µl of eluate was then incubated for half an hour with 20µl of protein-A beads crosslinked to anti-T7 antibodies. Non-immunoprecipitated material was then removed and retained. The beads were then washed twice in mRNP binding buffer, the buffer removed and a further 200µl added. Both immunoprecipitated and non-immunoprecipitated material was then RNA extracted as described in Methods and Materials, the first stage of which involved proteinase K addition serving to elute T7-Xp54/mRNA structures from the antibody-crosslinked beads. RNA pellets were then raised in 80µl of water, 2µl of which was used in RT-PCR reactions targeting mRNA for the following genes: ribosomal protein *L1* (*rpL1*), *histone B4*, *FRGY1*, *cyclin B1* and *mos*. The agarose gels showing the results of these reactions are shown in figure 16.

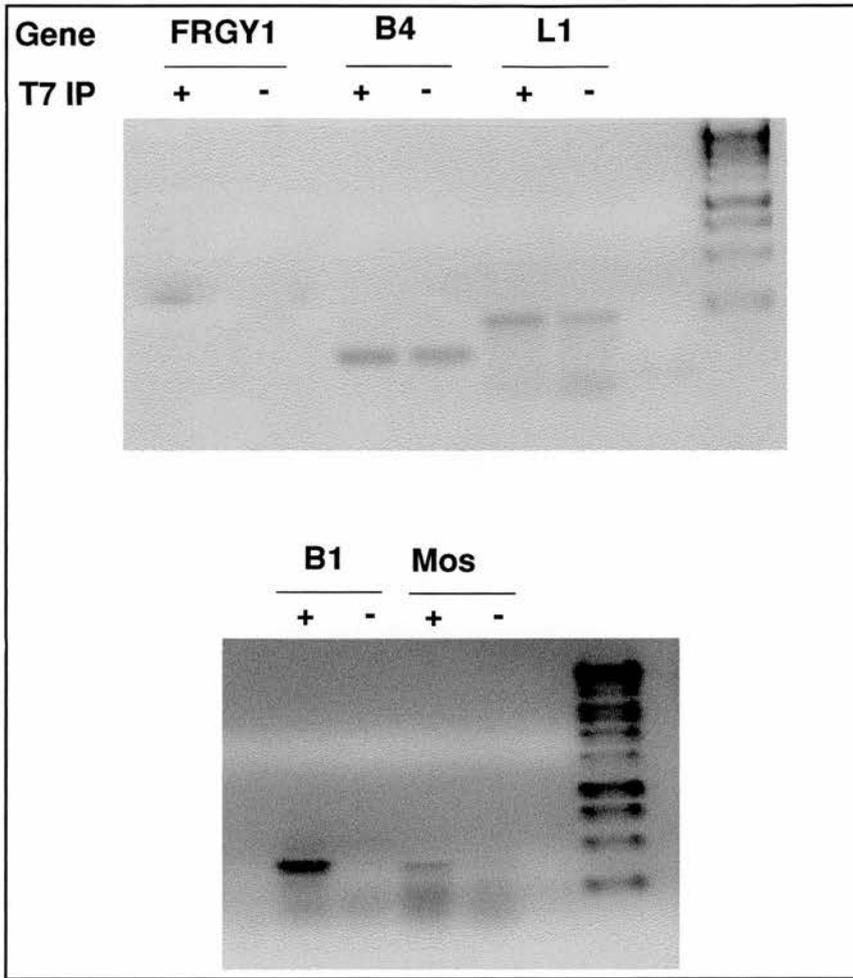


Figure 16: mRNAs differ in the degree of their association with Xp54-containing structures.

T7-Xp54 containing structures (+) were immunoprecipitated from polyA(+) material extracted from stage 4 oocytes. Both T7-Xp54 containing structures (+) and non T7-Xp54 containing structures (-) present in the polyA(+) material were RNA extracted and the RNA used as a template for RT-PCR reactions targeting ribosomal protein *L1*, histone *B4*, *FRGY1*, cyclin *B1* and *mos*.

Of the mRNAs analysed all show a detectable association with T7-Xp54 containing structures. *FRGY1*, *mos* and *cyclin B1* mRNA are only detectable in T7-Xp54 containing structures. *Ribosomal protein L1* mRNA and *histone B4* mRNA in contrast are present in both fractions of the poly(A)+ material in roughly equal amounts.

These results give an indication of which mRNAs are becoming associated with T7-Xp54 containing and thus presumably Xp54-containing structures at this stage (4) of oogenesis. It is not possible to determine which of those mRNAs analysed might not be associated with Xp54 from fig.16 but the high quantity of *cyclin B1* and *mos* mRNAs in the '+' fractions suggests these mRNAs are being incorporated into Xp54-containing structures in a significant quantity at stage 4 of oogenesis.

The lack of a detectable *FRGY1* signal in the '-' fraction may be due to the extraction procedure for some reason selecting poorly for the *FRGY1* message leaving a weak signal in the '+' fraction and nothing visible in the '-' fraction. The greater '+' *FRGY1* signal however still suggests that the greater proportion of its mRNA is becoming incorporated into Xp54-containing structures at this stage of oogenesis rather than being already incorporated although the instability indicated by figure 15 suggests there

is not yet a significant quantity of the message that is masked at this stage.

Overexpression of Xp54 differentially affects nuclear export of mRNAs

In order to gain an initial insight into the influence of Xp54 on various genes in the oocyte, stage 4 oocytes were either injected in the nucleus with a plasmid vector bearing recombinant forms of the Xp54 gene or were left uninjected. As part of the vector, either T7-tagged wild-type p54 DNA was injected or a T7-tagged form of the gene in which the Xp54 DNA had been mutated so as to produce an amino acid substitution in the DEAD-box motif of Xp54, a motif essential to the functioning of the RNA helicase (de la Cruz *et al.*, 1999), resulting in the motif DQAD. The latter involves a substitution from glutamic acid (E) to glutamine (Q).

The three sets of oocytes were incubated at room temperature for 24 hours or 48 hours and at both time intervals 30 nuclei and 15 cytoplasms were extracted from the injected oocytes and from the uninjected oocytes at 24 hours. This gave a total of 10 extracts: uninjected (nuclear and cytoplasm); wild-type (nuclei and cytoplasms) and DQAD (nuclei and cytoplasms) at 24 hours; the same wild-type and DQAD extractions at 48 hours. Fractions of both nuclei and cytoplasms were then prepared for western blotting and RNA extraction.

In order to prepare nuclei for SDS-PAGE analysis, the nuclei of each sample were collected in 20 μ l water and then were made up to 100 μ l in volume with DEPC-treated water and homogenised. A fraction of required volume was then taken for loading onto the gel as described in Methods and Materials.

To extract cytoplasmic proteins, cytoplasm samples were collected in 80 μ l water, homogenised with addition of 100 μ l of RNA extraction buffer and then treated with 100 μ l of freon to extract from lipids as described in Methods and Materials. A fraction of required volume was then taken for SDS-PAGE analysis as with nuclear samples.

The remainders of each sample of nuclei and cytoplasms were RNA extracted as described in methods and materials, to prepare RNA for RT-PCR analyses described below. RNA pellets were raised in 20 μ l of water giving an approximate concentration of 25 nuclei and 13.5 cytoplasms per RNA sample.

Protein samples were analysed for differences in nucleus/cytoplasm distribution and thus transport of the Xp54 variants. RNA samples were analysed by RT-PCR to detect any noticeable difference in export of specific mRNAs caused by synthesis of the Xp54 variants.

In order to see an accurate indication of the proportion of protein present

per nucleus and cytoplasm, immunoblotting was carried out loading quantities to give a volume equivalent to a 1:1 ratio of nuclei:cytoplasms. SDS-PAGE gels were analysed by western-blotting with mouse anti-T7 antibodies and two exposures of the blot were made, one after 2 minutes and the other after 30 minutes (figs.17(a) and (b) respectively).

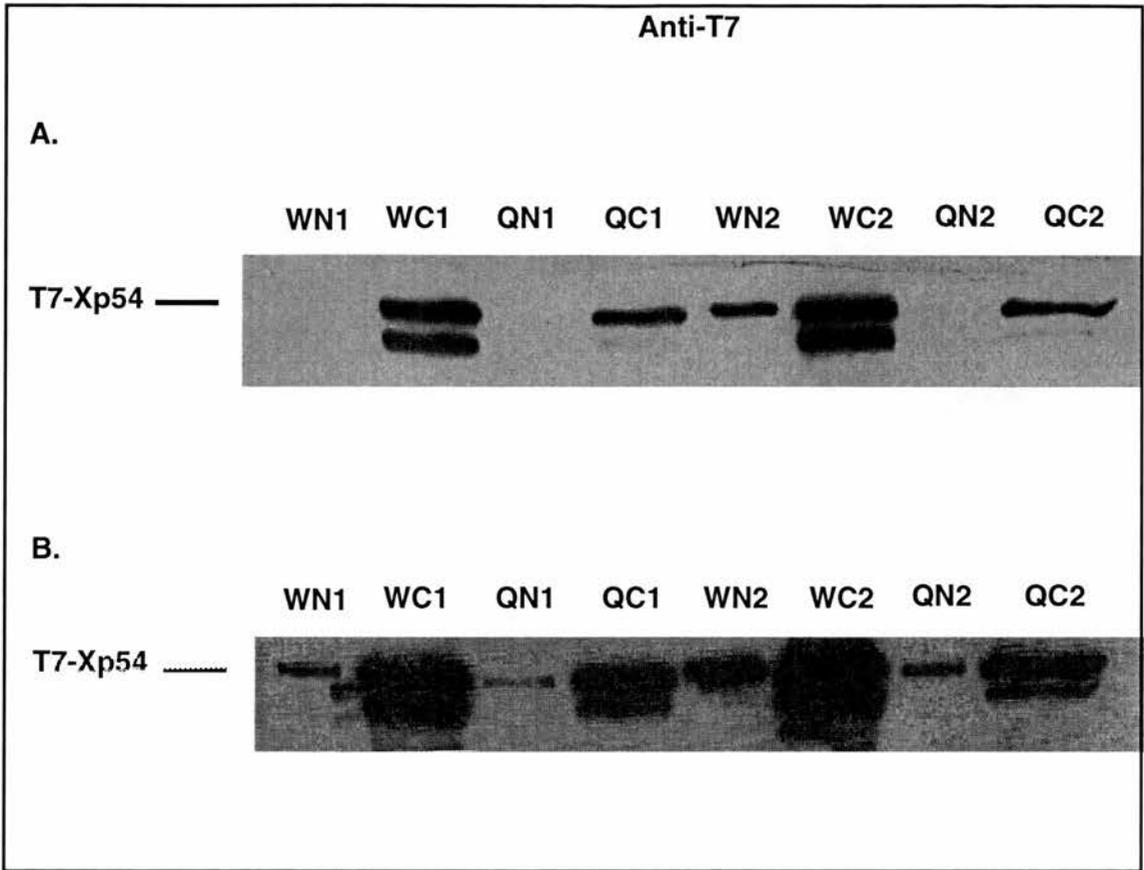


Figure 17: Nucleocytoplasmic distribution of recombinant forms of Xp54 expressed from plasmid vectors injected into the nucleus is disrupted by the DQAD mutation.

SDS-PAGE and immunoblotting was performed on nuclear and cytoplasmic samples extracted from oocytes injected with wild-type and DEAD-box mutant forms of Xp54-encoding DNA. (A) Exposure time two minutes; (B) exposure time thirty minutes.

Abbreviations: WN1: wild-type Xp54-injected, nuclei 24 hours incubation; WC1: wild-type Xp54-injected, cytoplasm 24 hours; QN1: DEAD-box injected oocyte, nuclei 24 hours; QC1: DEAD-box injected oocyte, cytoplasm 24 hours; the lanes suffixed with '2' correspond to cytoplasm and nuclei from wild-type and mutant Xp54-injected oocytes extracted at 48 hours.

Uninjected samples not shown and showed no detectable signal.

The samples show significantly lower levels of Xp54 protein in the nucleus compared to the cytoplasm but the protein is nevertheless present in both compartments in wild-type or DEAD-box mutant form. This indicates that the DQAD mutation has not prevented all export of the protein and does

not inhibit import. An increase in synthesis of each T7-tagged protein can be seen between the two time points. The reason for the apparently greater expression of the wild-type form of the tagged-protein at both time-points may be due to a higher concentration of DNA in the wild-type Xp54-expressing plasmid solution compared to the solution containing DQAD-Xp54 expressing plasmid and thus a greater amount of wild-type *Xp54* DNA micro-injected. It is not known however what the lower band in each cytoplasmic sample represents.

In both diagrams it can be seen that the wild-type Xp54 protein accumulates in the nucleus over 2 days faster than the DEAD-box mutant. The DEAD-box Xp54 protein is also expressed less in the cytoplasm than the wild-type form. The results suggest that the intracellular transport of Xp54 is disrupted by a DEAD-box mutation. It is not clear what causes the occurrence of the fragment seen in cytoplasmic samples but it would be expected to comprise much of the recombinant Xp54 protein starting from the T7 tag.

By attempting to overexpress Xp54 using an exogenous source of DNA to encode the protein, it is necessary to look at the effect this may have on endogenous Xp54 levels to ensure there is not such competition between expression-vector DNA/mRNA and endogenous *Xp54* DNA and mRNA

for expression factors such as translation machinery so that, in effect, the greater than normal expression of the protein is counteracted. Therefore stage 4 oocytes were nuclear-injected with either T7-*Xp54* expression vector, incubated over a range of times, protein extracted and then immunoblotted with antibodies recognising either T7 or Xp54 (and expectedly T7-Xp54). Protein was extracted from sets of 10 oocytes at 0, 12, 24 and 48 hours post T7-*Xp54* injection and an equivalent of 0.5 oocytes was loaded for each sample onto the SDS-PAGE gel. Densitometry was subsequently performed on the relevant bands in order to obtain a figure representing their relative volume that could be plotted graphically. The results are presented in figure 18.

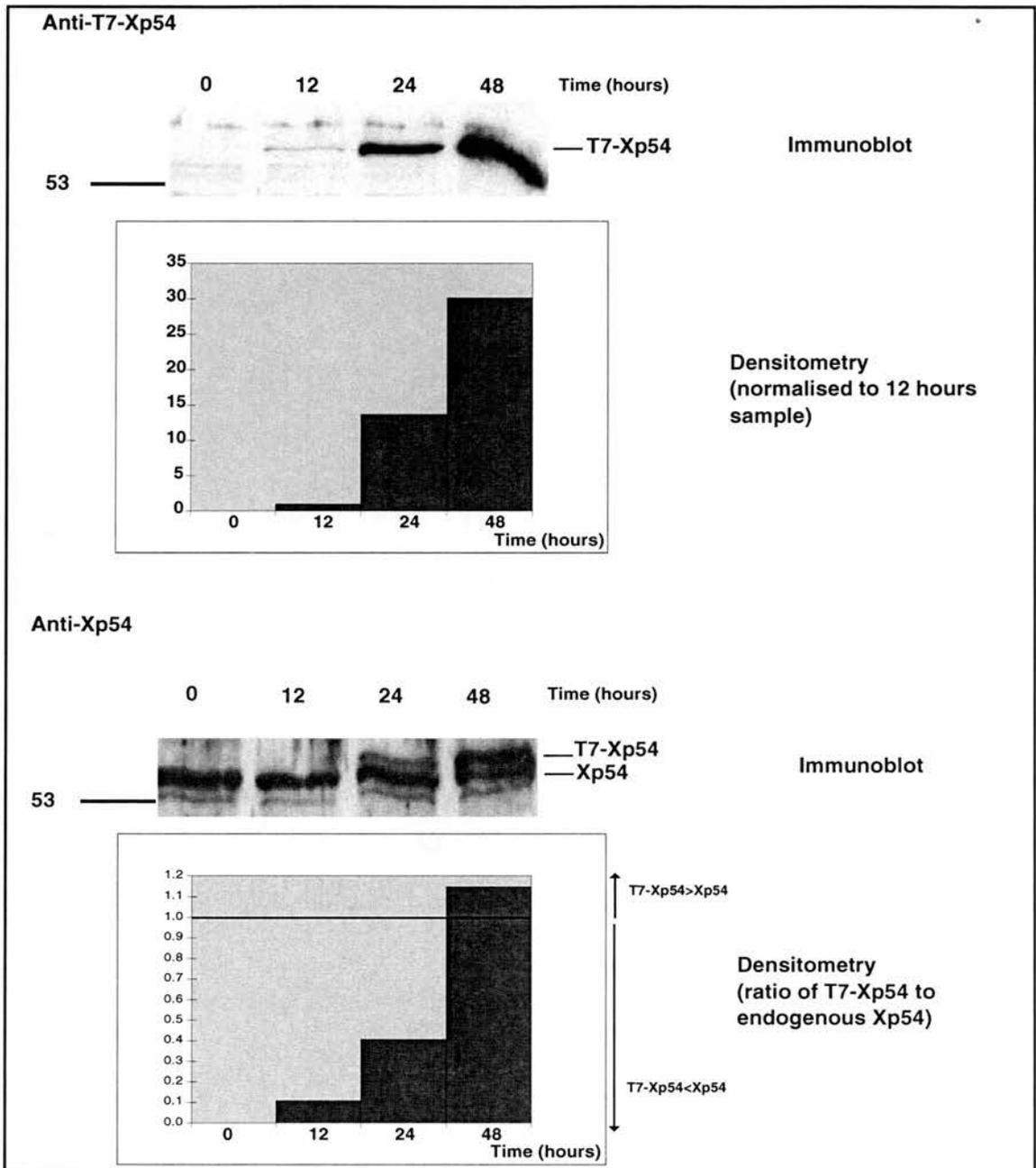


Figure 18: Expression of T7-Xp54 adds to the steady-state levels of oocyte Xp54.

Protein samples extracted from stage 4 oocytes, nuclear-injected with T7-Xp54, were separated by SDS-PAGE and immunoblotted with anti-T7 antibodies (top) or anti-Xp54 antibodies (bottom). Immunoblots are shown with lanes corresponding to the time in hours following microinjection that protein was extracted. Molecular size marker positions (KDa) are shown on the left-hand side. Also shown is the position of endogenous Xp54 and/or the position of T7-Xp54.

Densitometry was performed on the bands for either Xp54 or T7-Xp54 using GeneTools™ software (Synoptics Ltd) and the resulting figures presented below graphically. Values for the anti-T7 immunoblot are normalized to time 12 hours sample given a value of 1 and values for the anti-Xp54 immunoblot are presented as a ratio of T7-Xp54 : Xp54 densitometry.

Figure 18 shows that whilst T7-Xp54 accumulates rapidly, the level of endogenous Xp54 remains relatively steady, only fluctuating to a small degree during the course of 48 hours. This tallies well with later results which show endogenous Xp54 has a relatively stable presence across this time-frame. The increasing T7-Xp54 presence is consistent with the increased synthesis of T7-Xp54 seen between the two timepoints in figure 17. The lower immunoblot also shows that the anti-Xp54 antibody also recognises the T7-Xp54 protein but binding is presumably stronger to endogenous Xp54 which appears to reduce the extent of binding of the antibody to T7-Xp54. The pattern of T7-Xp54 accumulation revealed by the anti-Xp54 immunoblot remains similar however and enables a densitometry ratio of T7-Xp54 to Xp54 to be taken as shown in the lower histogram in fig. 18. This indicates that whilst T7-Xp54 is barely detectable at 12 hours, it exceeds the quantity of endogenous protein by 48 hours.

A gel analysis was initially performed on the RNA samples. 0.5µl of nuclear RNA and 2µl of a 1:10 dilution of cytoplasmic RNA were loaded onto a 1.5% agarose gel. This gave an approximate ratio of 0.3 nuclei to 0.14 cytoplasms (2.5 to 1 nuclear:cytoplasmic RNA), the cytoplasmic RNA being diluted in 10µl water. The results are shown in figure 19(a).

The two visible bands present in most of the 10 lanes loaded represent the most prevalent two sizes of ribosomal RNA in oocytes and are taken to be an indicator of total RNA levels in the oocytes. Intensities of RNA vary slightly between the extractions corresponding to the different treatments which is noted below and as expected between nuclear and cytoplasmic samples given that equivalent amounts of protein can be observed only in a 5:1 ratio of nuclear:cytoplasmic extracts in stage 3 oocytes (pers. comm., J.Sommerville).

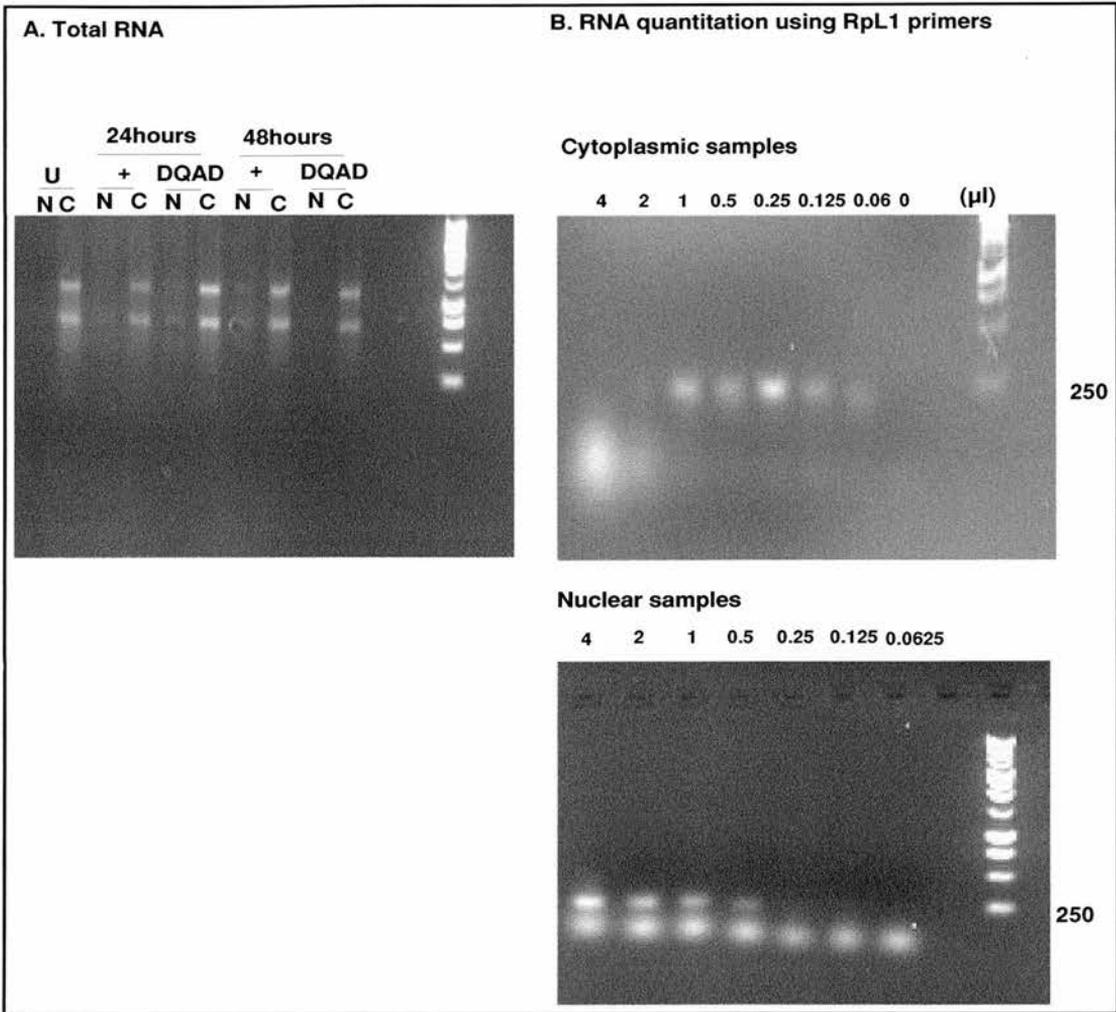


Figure 19: RNA gel and quantitative RT-PCR gels for *Xp54* DNA-injected oocyte samples.

(A) Lanes correspond to nuclear (N) and cytoplasmic (C) RNA samples from injected oocytes. Oocytes were either uninjected (U) or injected with wild-type *Xp54* DNA (+) or *Xp54* DNA bearing the DQAD mutation (DQAD). Injected oocytes were collected at 24 hours or 48 hours..

(B) Values shown above the gels are the quantities (in μl) of RNA template used in the RT-PCR reaction. Markers are shown on the rightmost position of the gel and the size of the lowest marker is given. The *L1* fragment is located at approximately the same vertical position as the lower marker; excess primer and RNA is visible below the amplified fragment in most lanes.

Also shown in fig.19 is a RT-PCR product calibration done to demonstrate that the technique is quantitative in that the intensity of the resulting product when visualized on a gel is dependent on template quantity. Various quantities of RNA template from the wild-type *Xp54*-injected oocytes' nuclei and cytoplasms were used in RT-PCR reactions to amplify the *ribosomal protein L1* mRNA. The lowest quantities are effective values and were achieved by using 10x the quantity shown of a 1:10 dilution of the RNA. The primers were designed to produce a fragment size of 240 base-pairs (bp). In both nuclear and cytoplasmic samples there is a general trend towards a decrease in product quantity with decreasing template until there is no visible product at the smallest template quantities. The lack of product at the highest cytoplasmic quantities is thought to represent an inhibitory effect of excessive RNA on the reverse transcriptase and/or polymerase enzyme.

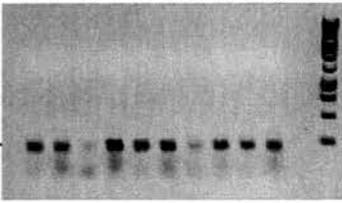
The RNA for each of the 10 prepared samples was then used as a template for RT-PCR reactions in order to amplify specific mRNAs and gain a quantitative reflection of the levels of each mRNA in each sample as judged by the intensity of the final, amplified cDNA product. A volume of 0.5 μ l of RNA was added to each RT-PCR tube. This is equivalent to

approximately 1 nucleus and 0.5 cytoplasms that were eventually loaded onto the 2% agarose gel. Several genes were analysed by this method and the resulting gels are shown in fig.20(A) and fig.21. All primers were based upon publicly available *Xenopus laevis* sequences in the EMBL databank whose functions are described in Appendix A.

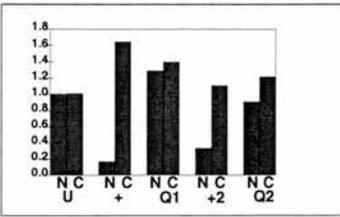
A.

RpL1

U		24hours				48hours			
		+		DQAD		+		DQAD	
N	C	N	C	N	C	N	C	N	C

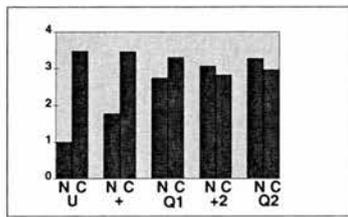
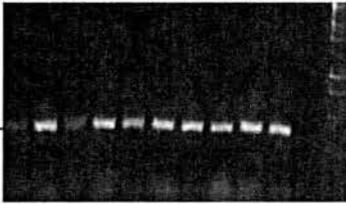


mRNA export (normalised densitometry)



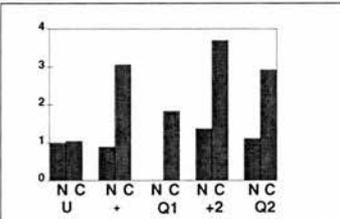
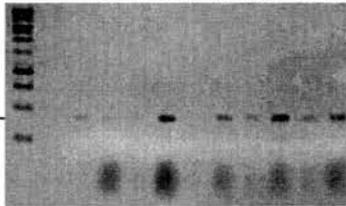
RpS1

U		24hours				48hours			
		+		DQAD		+		DQAD	
N	C	N	C	N	C	N	C	N	C



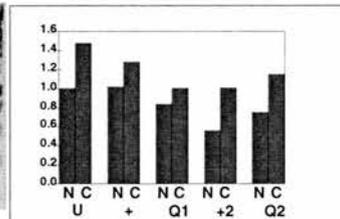
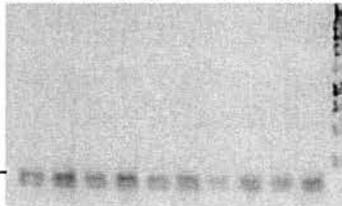
Cyclin B1

U		24hours				48hours			
		+		DQAD		+		DQAD	
N	C	N	C	N	C	N	C	N	C



Histone B4

U		24hours				48hours			
		+		DQAD		+		DQAD	
N	C	N	C	N	C	N	C	N	C



B.

Total mRNA stability (normalised densitometry)

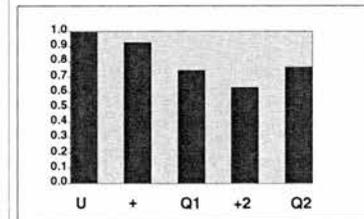
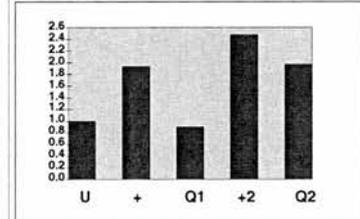
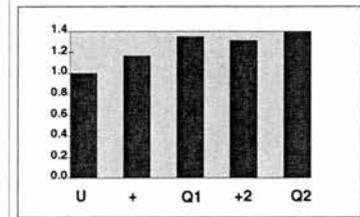
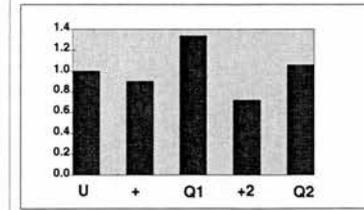


Figure 20: (A) Overexpression of Xp54 enhances nuclear export of selected mRNAs (*rpLI*, *rpSI*, *cyclin B1*).

Densitometry of RT-PCR product gel bands was undertaken using GeneTools analysis software (Synoptics Ltd) which measures the relative volume of the the graphical peak representing the intensity measurement of each band. Densitometry values were plotted relative to a normalized value of 1 for the nuclear/uninjected samples.

(B) Overexpression of wild-type Xp54 tends to destabilise those mRNAs translated during oogenesis (*rpLI*, *rpSI*) and stabilise those stored until after oocyte maturation (*cyclin B1*) relative to overexpression of mutant Xp54.

Densitometry was done as (A) but the nuclear and cytoplasmic values were added together and then normalized against a combined uninjected sample value of 1.

Positions of RT-PCR products for the target genes are indicated by black bars.

Abbreviations: U: uninjected; +: wild-type Xp54 DNA injected; Q: DQAD-mutated Xp54 DNA injected; N: nuclear; C: cytoplasmic. Samples taken at 24 hours(1) and 48 hours(2).

Certain RT-PCR analyses suggest an enhancement of nuclear mRNA export when exogenous Xp54 is expressed in the oocyte. This is visible for *rpL1*, *rpS1* and *cyclin B1* as a higher cytoplasmic value in the wild-type lanes relative to the uninjected cytoplasmic sample and the DQAD-injected cytoplasmic samples (with the exception of the second wild-type cytoplasmic samples for *rpL1* and *rpS1*). Also and perhaps more importantly, for *rpL1* and *cyclin B1* and the first time point for *rpS1* there is a higher cytoplasmic:nuclear densitometry difference, and so higher ratio, in the wild-type injected samples relative to the uninjected and the DQAD-injected. The *histone B4* analysis however did not show clearly either of these features which would have suggested an enhancement of nuclear export of *histone B4*. Certainly, differences between *histone B4* nuclear and cytoplasmic densitometry values in the wild-type injected samples are not as clearly different to the other samples as they are for *rpL1*, *rpS1* and *cyclin B1*.

There is also evidence suggesting that the DQAD mutation inhibits the clearing of the mRNAs from the nucleus that the exogenous wild-type Xp54 apparently accelerates. This is seen mainly for *rpS1* and also *FRGY1* (fig.21, see below) from the smaller nuclear and cytoplasmic densitometry differences seen particularly in DQAD-injected samples when compared to

the differences seen in the uninjected sample.

The differences between cytoplasmic and nuclear values in the wild-type injected samples for *histone H4* do not clearly show any enhancement of mRNA export relative to the uninjected control. In fact both the wild-type injected and DQAD-injected samples suggest a slight inhibition of nuclear export of the *H4* mRNA relative to the uninjected although there are no clear, consistent ratio differences between each other indicating possibly an artefact of injection.

Taking into account the apparent under-representation of total RNA in the +1C and Q2N samples relative to other nuclear and cytoplasmic samples (fig.19a) would not appear to affect these interpretations significantly as most of the genes analysed above have significant representation in the +1C gel lanes and the Q2N lanes in the figure 20 gels are either higher or not drastically lower than the Q1N intensities.

Overexpression of Xp54 selectively destabilises and stabilises different mRNAs

After noting the apparent effect of injected forms of *Xp54* DNA on nuclear export of oocyte mRNAs, the same RNA samples were then used to attempt to examine the effect of the DQAD mutation and exogenous *Xp54*

expression on the net stability of oocyte mRNAs by comparing the total RT-PCR intensities, as determined by densitometry, for each sample. That is, the combined intensities of nuclear and cytoplasmic RNA bands per oocyte treatment were added together. RT-PCR analyses were performed for those genes described above — *rpL1*, *rpS1*, *cyclin B1*, *histone B4*, *histone H4* and *FRGY1*.

The combined and normalized densitometry values for these RT-PCR analyses are shown in fig.20(b) and fig.21.

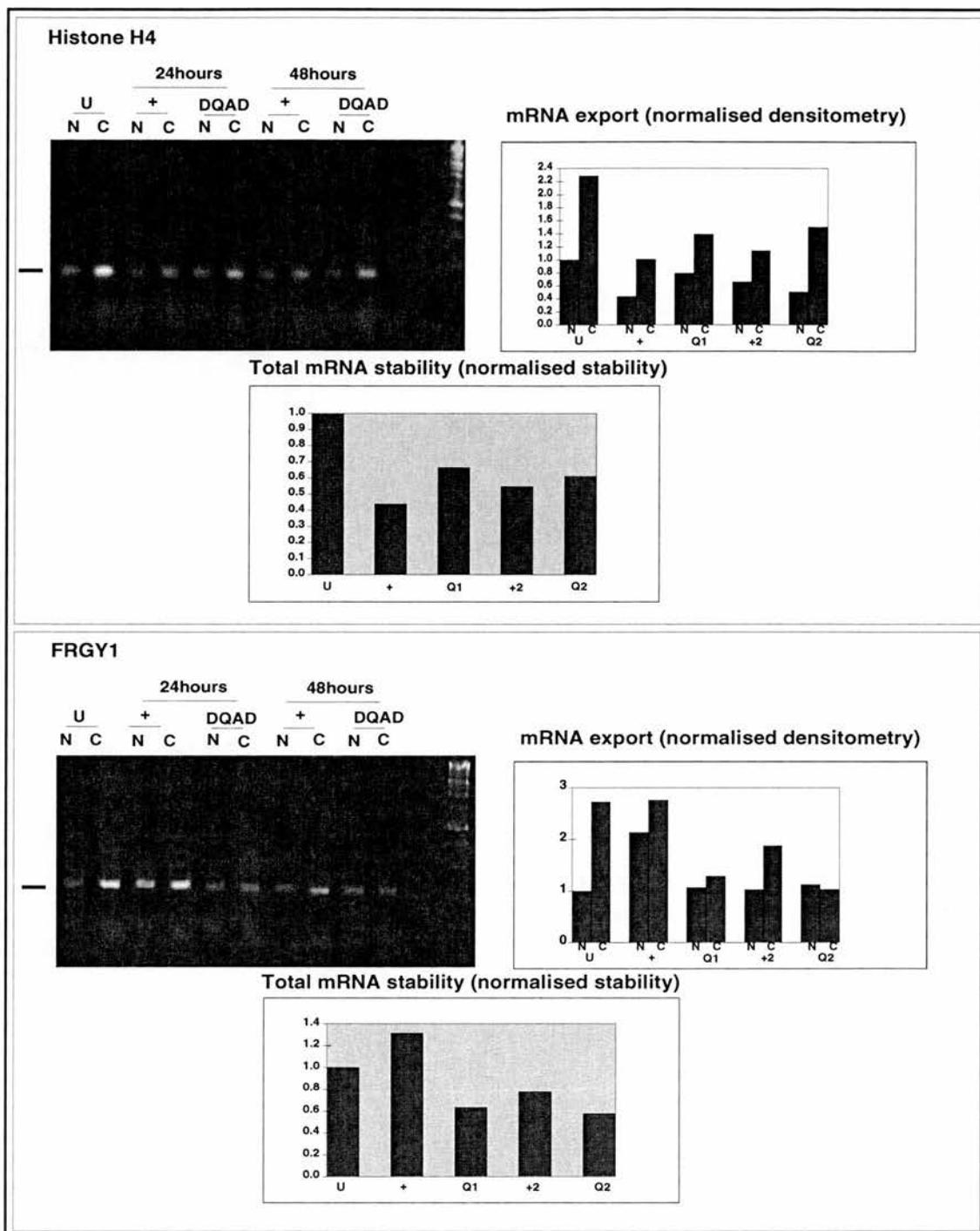


Figure 21: Overexpression of wild-type Xp54 tends to destabilise those mRNAs translated during oogenesis (*histone H4*) and stabilise those stored until after oocyte maturation (*FRGY1*) relative to overexpression of mutant Xp54.

Data on nuclear export of *histone H4* and *FRGY1* mRNA are also shown.

Densitometry of RT-PCR product gel bands undertaken as fig.20(B).

Positions of RT-PCR products for the target genes are indicated by black bars.

Abbreviations: U: uninjected; +: wild-type Xp54 DNA injected; Q: DQAD-mutated Xp54 DNA injected; N: nuclear; C: cytoplasmic. Samples taken at 24 hours(1) and 48 hours(2).

By comparing the combined nuclear and cytoplasmic densitometry values in each sample for the analysis of each gene, it was hoped to obtain a reflection of the net stability of the mRNA for each gene in each sample corresponding to each treatment or the uninjected state. That is, by comparing the values obtained from overexpressing Xp54 or overexpressing the mutated form with each other and with the uninjected values, possible influences on the synthesis (transcription) and/or degradation of the mRNAs by Xp54 can be inferred.

From looking at figs.20(b) and 21, it can be seen that the mRNA for *rpL1* and *histone H4* is apparently destabilised by exogenous wild-type Xp54 expression when compared to the DQAD-injected and uninjected samples. For *cyclin B1*, the reverse is true — the exogenous wild-type Xp54 expression has apparently stabilised the corresponding mRNAs relative to the uninjected and DQAD-injected treatment. The mRNAs for *histone B4* and *FRGY1* in contrast to *cyclin B1* — all understood to be non-translated genes at this stage — do not uniformly (*i.e.* relative to both DQAD-Xp54 injected and uninjected across both time-points) suggest stabilisation by exogenous Xp54 expression although *FRGY1* shows greater values for the wild-type Xp54 samples relative to the DQAD-injected. This could therefore indicate, rather than Xp54-induced stabilisation, DQAD-mutated

Xp54 induced destabilisation of the mRNA. The mRNA for *rpS1* may have been stabilised by overexpression of both forms of Xp54 and neither form appears to have resulted in any destabilisation relative to the uninjected sample (fig.20b).

The overexpression of Xp54 thus seems to bring about changes in the stability of mRNA for certain genes in contrast to the overexpression of the mutant form which seems to have the opposite effect for these genes. For other genes, the overexpression of either form seems to have varying effects on stability.

With regards to stability any under-representation (as is implied by figure 19(a) in the +1C and Q2N samples) might further reinforce the destabilisation by Xp54 overexpression hypothesis for *rpL1* and *histone H4* at the 48 hour interval. Under-representation of RNA in these samples would also be thought to slightly lessen the validity of the stabilisation by Xp54 overexpression hypothesis at the 48 hour time interval for *cyclin B1* alone. Similarly, for *FRGY1* the hypothesis of destabilisation by DQAD-Xp54 expression would be slightly weakened for the 48 hour interval by under-representation of the corresponding RNA sample as implied by figure 19(a).

An *Xp54* antisense morpholino inhibits *Xp54* mRNA translation and affects its stability

The nature of *Xp54*'s influence on oocyte gene expression was then examined by aiming to reduce its expression in the oocyte. This was achieved by injecting oocytes with an antisense morpholino oligonucleotide (GeneTools) designed to target *Xp54* mRNA (fig.22b). Morpholinos are designed to bind to the region around the translation start site in the mRNA and sterically block translation from initiating thus reducing the total amount of expression from a particular protein-coding gene in an injected cell. They have advantages over certain other antisense oligonucleotides (figure 22a).

Firstly, the occurrence of reduced *Xp54* was verified and examined by looking at the effect of the *Xp54* antisense morpholino's injection on recombinant *Xp54* protein levels and on the level of endogenous *Xp54* mRNA levels.

Stage 4 oocytes were nuclear or cytoplasmically injected with T7-*p54* vector alone or in a mixture with a concentration of 1nm/μl of *Xp54* antisense morpholino. The oocytes were left to stand at room temperature for 24 hours. This gave a total of 4 sets of samples. 20 cytoplasms from

each sample set were collected in 80 μ l of water, freon extracted as described in Materials and Methods and then 15 μ l of SDS-PAGE sample buffer added.

15 μ l of each cytoplasm sample was then loaded onto an SDS-PAGE gel. This corresponded therefore to approximately 2.5 cytoplasms. The resulting immunoblot for T7-p54 is shown in figure 23(a).

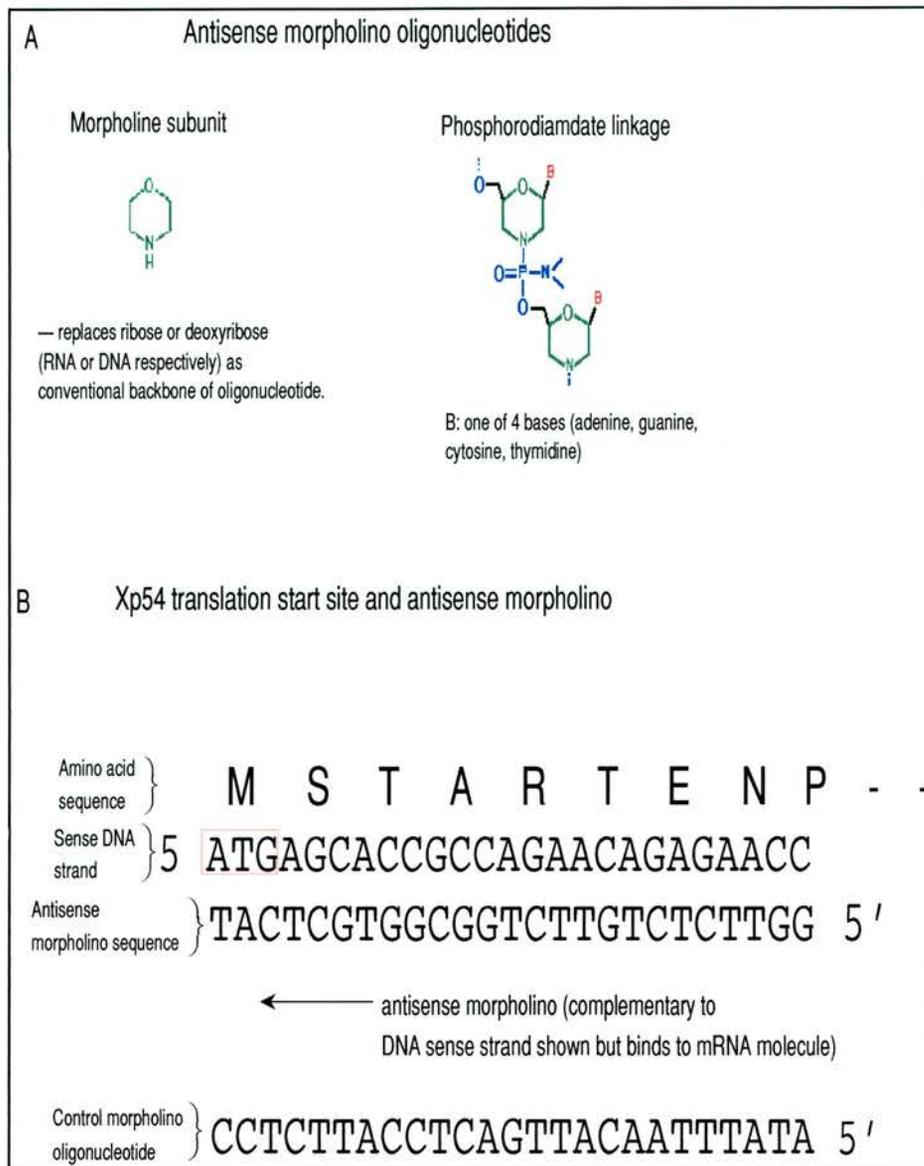


Figure 22: Xp54 Morpholino antisense oligonucleotide.

(A) The morpholino is a polymer of morpholine subunits which form the 'backbone' of the oligonucleotide in the way that ribose or deoxyribose subunits do in RNA or DNA. Advantages include nuclease resistance and high affinity to target sequences with the ability to bind to RNAs containing regions of secondary structure.

(B) The morpholino is designed to be complementary to the translation start site and must be no more than 30 nucleotides downstream of the start codon (red box) to inhibit translation initiation. The Xp54 morpholino is 25 nucleotides in length, the commercially recommended length.

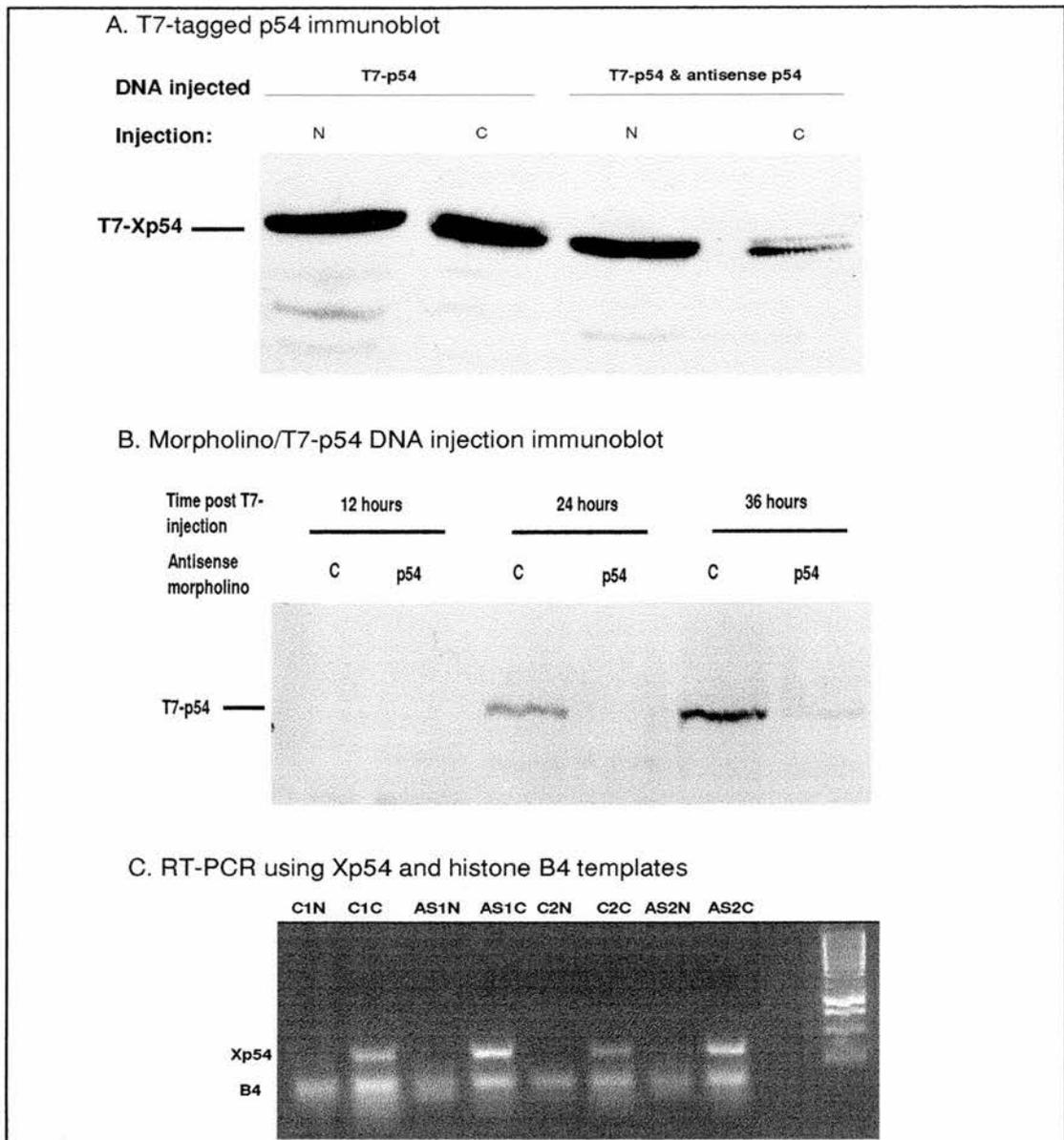
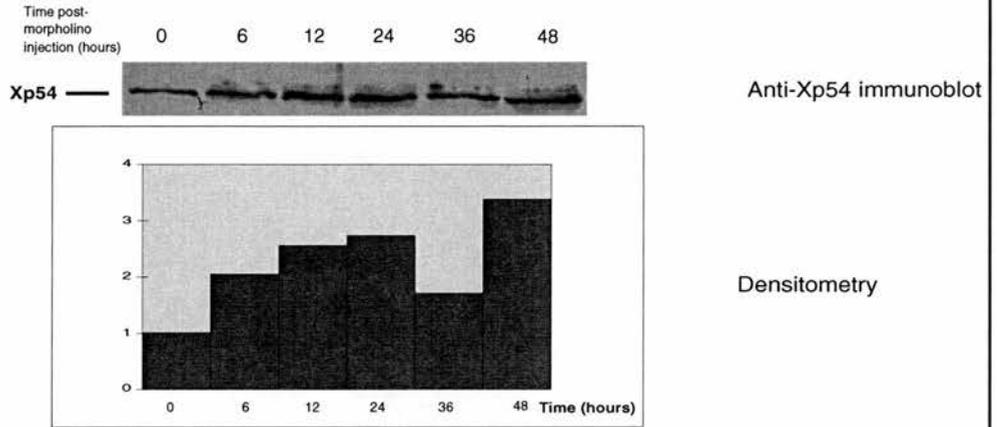


Figure 23 (a-c): An antisense *Xp54* morpholino inhibits *Xp54* mRNA translation and affects its mRNA stability.

Abbreviations: (a) N: sample of nuclear injected oocytes with T7-*Xp54* and/or anti-*Xp54* morpholino oligonucleotide; C: sample of cytoplasmically injected oocytes with T7-*Xp54* and/or anti-*Xp54* morpholino oligonucleotide. (b) C: control morpholino and T7-*Xp54* injected; p54: antisense *Xp54* morpholino and T7-*Xp54* injected (c) C(1/2)N: control injected (1st/2nd) nuclear samples; C(1/2)C control injected cytoplasmic samples; AS(1/2)N: antisense injected nuclear samples; AS(1/2)C: antisense injected cytoplasmic samples; markers are shown at rightmost position, the lowest two being 250bp and 500bp. The position of the *Xp54* band and B4 internal control band is indicated.

D. Endogenous Xp54

Control morpholino injections



Antisense morpholino injections

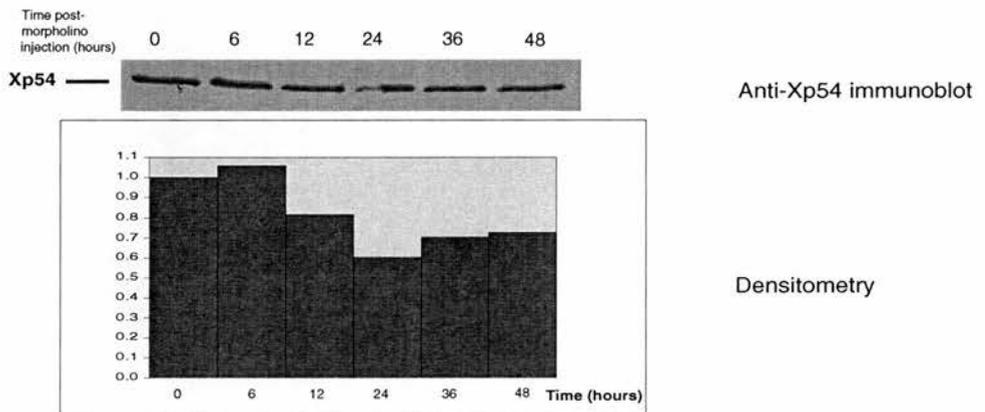


Figure 23(d): An antisense *Xp54* morpholino inhibits *Xp54* mRNA translation and affects its mRNA stability.

Protein was extracted from groups of 10 stage 4 oocytes injected with either the control or *Xp54* antisense morpholino at the times indicated post-injection. The immunoblots of the separated proteins show each time sample and the graphs below indicate the densitometry performed on the bands which is normalized to a value of 1 given to the first sample (time 0).

The immunoblot indicates a reduction in translation of exogenous *Xp54* mRNA brought about by the antisense morpholino directed against its sequence (fig.23a). Cytoplasms only were loaded as previous studies have indicated that stage IV oocyte nuclei have a very low proportion of the total cellular *Xp54* protein (Smillie and Sommerville, 2002). The effect of the antisense morpholino is significantly more pronounced in the cytoplasmically injected samples which is surprising as the morpholino might be expected to bind to the mRNA as it was being transcribed in the nucleus.

The level of endogenous *Xp54* protein following morpholino injection was also investigated. Stage 4 oocytes were injected with the same concentration as above of either the control morpholino or the antisense *Xp54* morpholino and then collected at the following times after incubation at room temperature: 0 hours, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours. 10 oocytes from either set were collected at each interval and homogenised with 80µl of RNA extraction buffer and then freon extracted as described in Methods and Materials before being subjected to SDS-PAGE and immunoblotting with a rabbit anti-*Xp54* antibodies (1:2000 dilution). A volume equivalent to approximately 1 oocyte was loaded per track of the gel. The resulting immunoblots (fig.23d) indicate contrasting

results between control- and antisense-injected oocytes with Xp54 apparently increasing in the former and declining after 6 hours in the latter to a level which remains less than the time 0 sample for at least 48 hours. The 36 hour interval in the control immunoblot may represent an aberration in the blotting or extraction procedure as might the 24 hour interval in the antisense immunoblot which might not be expected to be so low in intensity. Although a steady state of Xp54 levels might be expected under control conditions rather than a gradual accumulation, the contrasting reduction over time in Xp54 band intensity in the antisense-injected samples suggests that the antisense morpholino as a result of its sequence — designed to target *Xp54* mRNA — is able to make an impact on the total pool of cellular Xp54 after at least 6 hours, up until which point both control- and antisense-injected samples show an increase in Xp54. In order to see whether the sharp increase in endogenous Xp54 indicated by the control immunoblot in fig.23(d) might represent some kind of response to the microinjection process, the levels of endogenous Xp54 were also investigated in non-injected oocytes over the same time frame. Also Xp54 levels were also monitored in oocytes incubated with a concentration of 50µg/ml of cycloheximide over the same time span. With the translation inhibiting properties of cycloheximide it was hoped that a reduction of

general oocyte translation would be reflected by a reduction in the level of Xp54 detected after immunoblotting relative to the non-injected thus indicating whether part of the total Xp54 signal that should be obtained from non-injected oocytes arises from translation occurring subsequent to the onset of experimental timing. The results are shown in fig.23(e) with a volume of sample equivalent to approximately 0.5 oocytes loaded per lane of the gels.

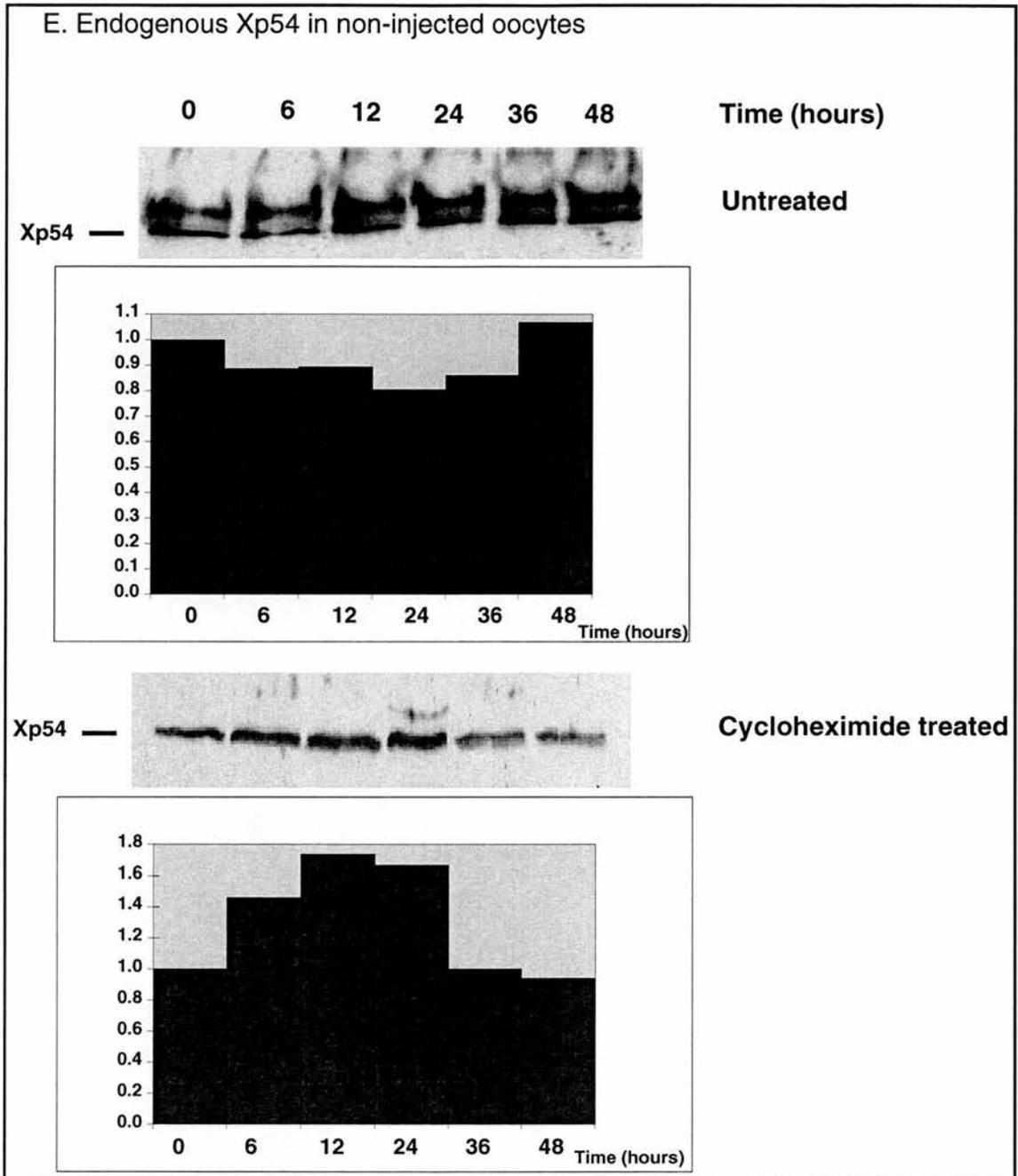


Figure 23(e)

Protein was extracted from groups of 10 stage 4 oocytes at the indicated times. Oocytes were either left untreated (non-injected) or incubated with 50 μ g/ml cycloheximide. The immunoblots of the separated proteins show each time sample and the graphs below indicate the densitometry performed on the bands which is normalized to a value of 1 given to the first sample (time 0). The lower band in each lane of the top gel corresponds to Xp54.

The non-injected oocytes in fig.23(e) exhibit a relatively stable net presence of Xp54 compared to the control-injected oocytes of fig.23(d). That is, a relatively steady state of Xp54 suggesting that the injection process may have been responsible for some response leading to an increase in Xp54 levels that was attenuated by the antisense morpholino but not by the control morpholino. In light of this, the antisense morpholino therefore still seems able to counteract the new translation of Xp54 that occurs following injection and this inhibition is visible after 6 hours in fig.23(d). It is not known what the upper band that is recognised by the rabbit anti-p54 antibody in the non-injected oocyte gel represents but the lower band in all cases corresponds to Xp54 being located just above the 53KDa size marker on the gel. The cycloheximide treated oocytes indicate that any reduction in translation takes over 12 hours to become visible following addition to the oocyte buffer. Xp54 levels apparently continue to increase for these 12 hours as in the first few time points of the control although even allowing time for the cycloheximide to diffuse into the oocytes it is not known why there should be an increase apparently as a response. The increase is not though to the same degree as in the control morpholino-injected oocytes and could partly represent a normal fluctuation in Xp54. After 12 hours post-treatment the drop in Xp54

quantity begins to become noticeable but by no means a complete inhibition of *Xp54* translation at the concentration of cycloheximide used. This suggests that normally *Xp54* does have limited stability within the time frame of the experiment degrading visibly after approximately 12 hours with the result that its total levels decline unless translation is occurring.

The time course of the morpholino's efficacy and effect on *Xp54* mRNA translation was also investigated using the T7-*Xp54* construct. Stage 5 oocytes were cytoplasmically injected with the antisense morpholino or control morpholino first and left at room temperature for 12 hours prior to a second injection with T7-tagged *Xp54* DNA. 15 oocytes were then collected from either set at 12, 24 and 36 hours. 50µl of water was added to the 15 oocytes before freon extraction and 30µl sample buffer. The quantity of sample loaded was approximately equivalent to 4 oocytes per lane of an SDS-PAGE gel. The resulting immunoblot is shown in figure 23(b).

The antisense morpholino can be seen to inhibit translation of the injected T7-*Xp54* mRNA effectively for almost 36 hours as the protein is only just visible at the third time interval. In contrast the random sequence of the control antisense morpholino permits a strong degree of translation from the mRNA of the T7-*Xp54* construct with protein being clearly visible at 24

hours post injection. Thus it is probably fair to say that the anti-*Xp54* morpholino significantly reduces translation from the injected T7-*Xp54* construct over the course of at least 36 hours.

If the anti-*Xp54* morpholino is influencing the translation of the target mRNA message as required it is reasonable to assume that there might be changes in the net stability of the message when compared to control morpholino-injected oocytes. Although morpholino oligonucleotides do not cause a message to be targeted for destruction but rely on impeding the access of translation machinery, any effects on net message stability would be expected to be indirect for example by preventing its translation and associated subsequent degradation. Stage 4 oocytes were cytoplasmically or nuclear injected with either the antisense or control morpholino oligonucleotide at a concentration of 1nm/μl. 50 nuclei and cytoplasm were then collected from each set of oocytes at 24 hours and 48 hours post-injection and freon and RNA extracted as detailed in Methods and Materials. 1μl of each of the resulting RNA preparations was then used in an RT-PCR reaction using primers designed to amplify a region of the *Xp54* mRNA. In addition primers to amplify a region of mRNA for the *histone B4* gene were included in the RT-PCR reactions to act as an internal control as preliminary experiments had shown that *B4* mRNA was

relatively unaffected by the injections performed in this study compared to most of the other genes analysed (data not shown). Thus the B4 signal should hopefully serve to indicate whether the experimental treatment has overtly affected the result of the reaction or that the reaction itself was faulty and indeed to help to show that the reaction had worked. Equal quantities of each reaction were then separated by agarose gel electrophoresis as shown in figure 23(c). Apparently low quantities of *Xp54* message are present in the nuclei (barely visible only in lanes C1N and AS2N) but the presence of the message is clear in all lanes corresponding to cytoplasmic samples. In both the 24 hour and 48 hour samples, the injection of the antisense *Xp54* morpholino has apparently resulted in an increase in the overall stability of the *Xp54* mRNA as indicated by its higher intensity than in control injected oocytes. It is then possible that in inhibiting translation of the *Xp54* mRNA, the morpholino may be preventing or delaying subsequent degradation of the message. Alternatively *Xp54* protein may normally interact with the mRNA for more of its own production so the presumed reduction in *Xp54* protein may exert a more complex indirect effect on the *Xp54* mRNA.

Injection of the *Xp54* antisense morpholino affects stability of different mRNAs in different ways

The influence of *Xp54*'s overexpression on the stabilities of various oocyte mRNAs has been seen and investigations were then carried out to determine the effect on specific mRNA stabilities of reduced *Xp54* protein expression induced by the *Xp54* antisense morpholino.

Stage 4 oocytes were injected with the *Xp54* antisense morpholino or the control morpholino of random sequence. 20 oocytes were then RNA extracted at the following time intervals post-injection: 0 hours, 6 hours, 12 hours, 24 hours and 36 hours and 48 hours. The control morpholino-injected oocytes were only sampled at 0 hours and 48 hours for comparison to the antisense-injected samples at the two extremes of the experiment. A quantity of 1µl of RNA from each sample in 9µl water was run on a 1.5% agarose gel to ensure equivalent quantities of RNA in each sample. RNA from the samples was then used in RT-PCR reactions, again amplifying the mRNAs of a range of genes to gain an impression of the relative quantities of their respective messages over time under control conditions and under the reduced *Xp54* conditions brought about by antisense injection. The agarose gels (2%) showing the products of the reactions for the genes

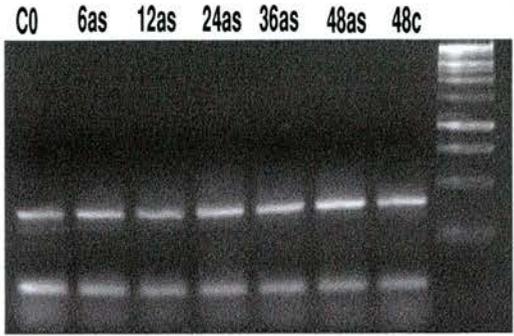
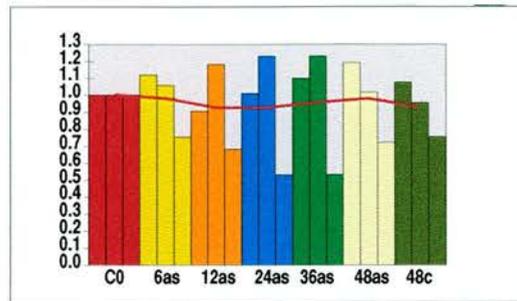
cyclin B1, *mos*, *FRGY1*, *ribosomal protein L1* and *histone B4* are shown in figure 24 along with histograms representing densitometry for three runs of the reaction. The gels shown constitute sets of reactions representative of the change in the mean densitometry value for that particular gene. Again primers for *histone B4* were included in all reactions as an internal control. The results of the analyses for the 5 genes shown in figure 24 indicate a pattern of mRNA quantity change that varies according to the gene under investigation. Also the histograms show several instances of a quite large degree of variability in the magnitude of the densitometric values between repeats of the RT-PCR analyses for each gene. However the pattern of change between each of the samples for an individual set of RT-PCR reactions (C0, 6as, 12as, 24as, 36as, 48as, 48c) is quite well conserved between the three runs with the occasional exception for example the changes in densitometry value between the early time point samples (6as and 12as) in the first run of the *FRGY1* and *mos* analyses. Also, although the pattern of change is well conserved in general, comparisons between antisense-values and the first control values are difficult for *rpL1* and *FRGY1* due to the presence of one set of values in each where the first control is uncharacteristically low giving a set of values normalized higher than the other two — a skewing in the normalization. Such anomalies are

thought likely to be down to experimental error particularly for example in quantity of loading in RT-PCR reactions and loading of gels. Such an error in loading a C0 sample for example would account for the skewing in normalization of the other results for that run of the experiment relative to the other runs of the experiment for that gene as all values are normalized to the C0 value, though the pattern of densitometry change between all samples other than C0 would not be affected.

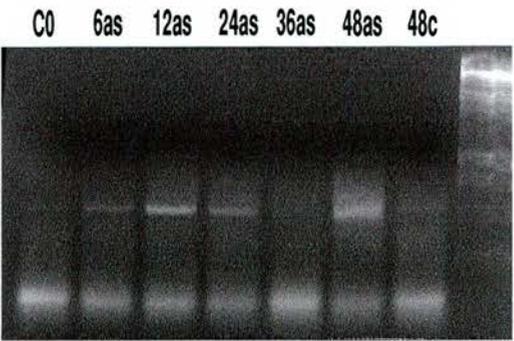
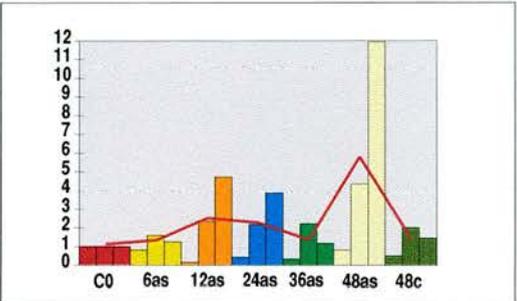
The mean change reveals that *cyclin B1* mRNA is relatively stable throughout the experimental period and unaffected by antisense morpholino targeting of Xp54. The mRNA for the other 4 genes in figure 24 show apparently greater changes in their relative presence within the oocyte as judged by RT-PCR analysis. However it cannot be taken as certain that these changes are largely brought about as a consequence of antisense injection as they may largely represent a normal pattern of change in that mRNA quantity. Equally though, the apparent trends such as reduction followed by rise in mRNA stability seen with *FRGY1*, *histone B4* and *rpL1* could represent an indirect consequence of a reduction in Xp54 protein brought about by the antisense morpholino treatment followed by a gradual wearing off of the morpholino's action assuming the mRNAs for these genes associate and interact in some way with Xp54.

Thus with regards to determining the existence of any possible consequence of morpholino injection on a gene's mRNA stability it is probably better to look at the relative mRNA levels at time points for which there are control injections — 0 hours and 48 hours. Consequently further analyses were then performed to supplement the data presented in figure 24. Using the RNA extracted from nuclei and cytoplasms at 24 hours and 48 hours post-injection from control- or antisense morpholino- (cytoplasmically) injected stage 4 oocytes (as used in fig.23c), RT-PCR reactions were performed with primers for *histone deacetylase (HDAC)* mRNA, *histone H4* mRNA or *ribosomal protein S1 (rpS1)* mRNA (figure 25). The densitometry obtained from the agarose gel analyses of these reactions are shown and include separate nuclear and cytoplasmic values which were added to give a combined reflection of mRNA quantity in the oocyte. This includes a control and antisense morpholino-injected quantity at 48 hours for comparison to the results in figure 24. Given that it seems likely that the antisense morpholino can have an effective inhibitory effect on *Xp54* mRNA translation for 48 hours post oocyte injection (figure 23b), an examination of the results at 48 hours is thought likely to be informative.

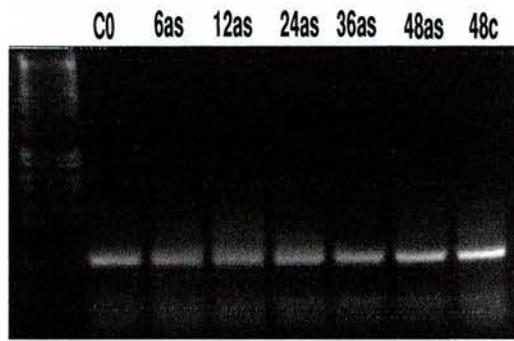
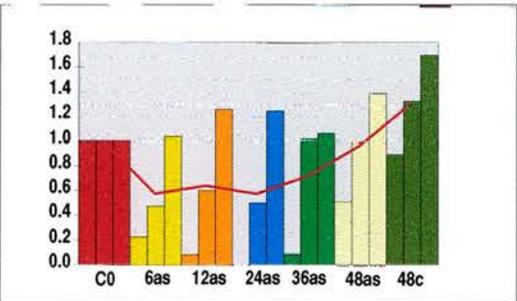
Cyclin B1/B4



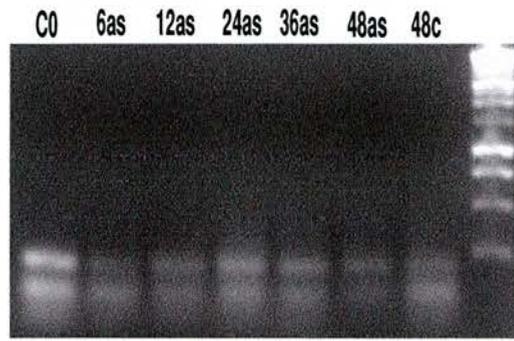
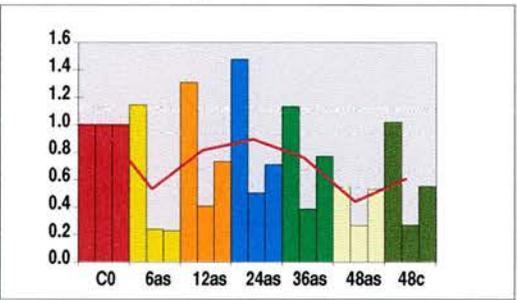
Mos/B4



FRGY1/B4



Ribosomal protein L1/B4



Histone B4

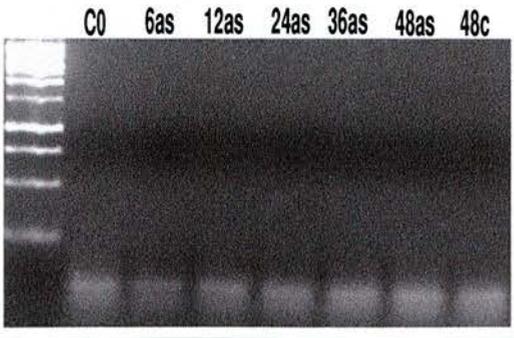
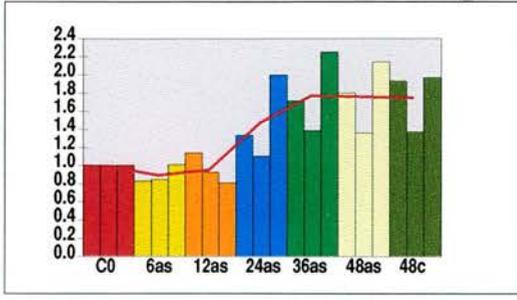


Figure 24: Injection of the *Xp54* antisense morpholino affects stability of mRNAs of different genes over time in different ways.

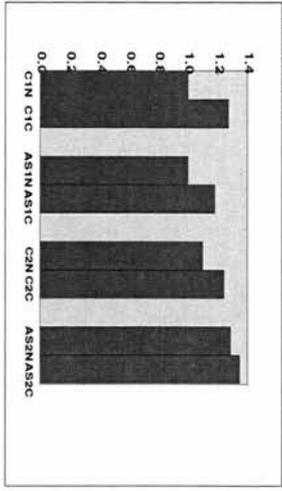
Histograms show densitometry values for three repeats of the RT-PCR reaction for each sample per gene. Each of the 7 RT-PCR reactions per run were performed concurrently in the same thermal cyclers. Densitometry values are normalized to a control value set at 1. Red line indicates change in mean value of normalized densitometry per sample.

Size markers are shown on left hand side or right-hand side for gels. Lowest four sizes are 1kbp (bright), 750bp, 500bp, 250bp. The *B4* internal control is the lower band in each of the seven labelled lanes per gel.

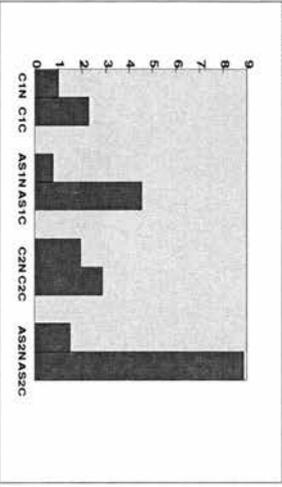
Abbreviations (histograms and gels): C0: control-injected at time 0 hours; 6as: antisense-injected at 6 hours; 12as: antisense-injected at 12 hours ;24as: antisense-injected at 24 hours ; 36as: antisense-injected at 36 hours ; 48as: antisense-injected at 48 hours ; 48c: control-injected time 48 hours

Nuclear-cytoplasmic mRNA distribution (normalised densitometry)

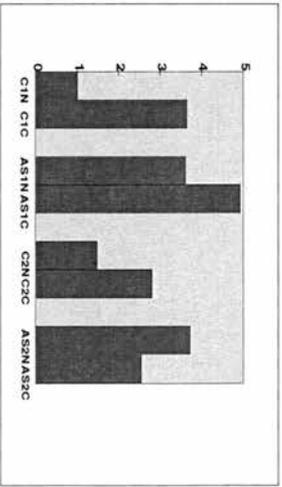
HDAC/B4



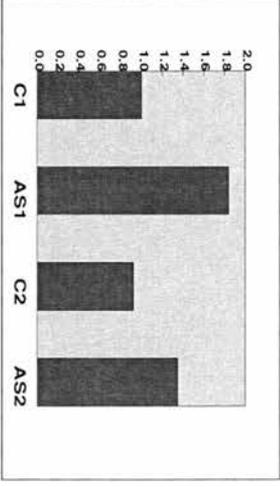
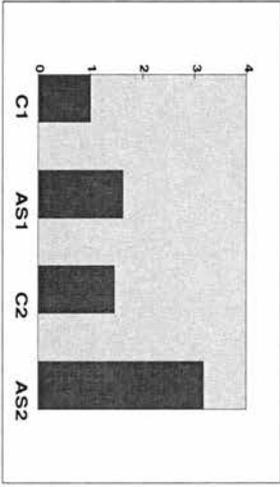
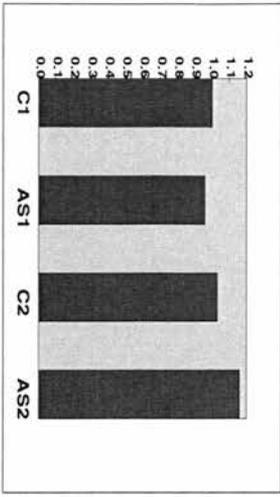
H4/B4



S1/B4



Relative total mRNA stability (normalised densitometry)



RTPCR gels

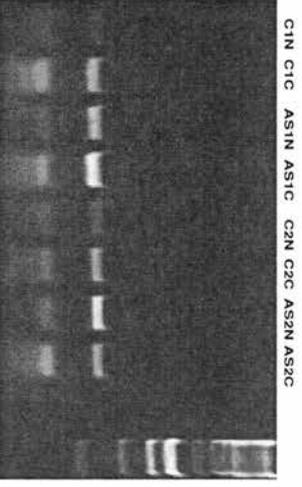
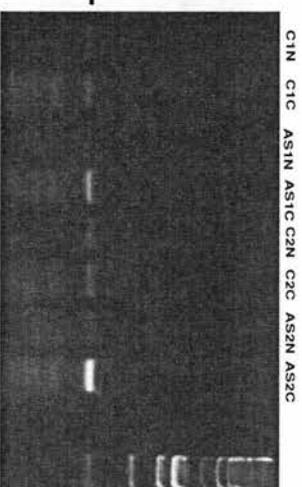
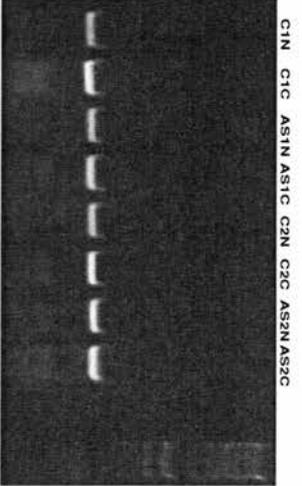


Figure 25: Injection of the *Xp54* antisense morpholino affects stability of mRNAs of different genes over time in different ways.

RT-PCR reactions were performed on RNA extracted from the nuclei and cytoplasm of stage 4 oocytes injected with the control or the antisense morpholino as described for figure 23(c). Histograms show the densitometry of the agarose gels (bottom row) normalized to the 1st control-injected nuclear samples (upper row) and show the combined nuclear and cytoplasmic densitometry (middle row) normalized to the 1st control samples.

Size markers are shown on left hand side or right-hand side for gels. Lowest four sizes are 1kbp (bright), 750bp, 500bp, 250bp. The B4 internal control is the lower band in each of the eight labelled lanes per gel.

Abbreviations (histograms and gels): C1N: nuclei of control-injected oocytes at 24 hours; C1C: cytoplasm of control-injected oocytes at 24 hours post-injection; AS1N: nuclei of antisense-injected oocytes at 24 hours; AS1C: cytoplasm of antisense-injected oocytes at 24 hours; C2N: nuclei of control-injected oocytes at 48 hours; C2C: cytoplasm of control-injected oocytes at 48 hours; AS2N: nuclei of antisense-injected oocytes at 48 hours; AS2C: cytoplasm of antisense-injected oocytes at 48 hours.

C1: combined nuclei and cytoplasm of control-injected oocytes at 24 hours; AS1: combined nuclei and cytoplasm of antisense-injected oocytes at 24 hours; C2: combined nuclei and cytoplasm of control-injected oocytes at 48 hours; AS2: combined nuclei and cytoplasm of antisense-injected oocytes at 48 hours.

The gene whose mRNAs seems to show notable antisense morpholino-induced stabilisation at 48 hours is *mos* (fig.24). Also the antisense morpholino seems to have a similarly clear effect on the *histone H4* and *rpS1* mRNA (fig.25). A seemingly clear destabilising influence by the antisense morpholino is seen for *FRGY1* at 48 hours post-oocyte injection (fig 24). These are the 4 mRNAs for which the control-comparable changes are most notable. It should be stressed however that it seems clear that in an oocyte the quantity of a gene's mRNA fluctuates through the course of 48 hours and whether this is due to the presence of the antisense morpholino or whether it is a natural process, the antisense morpholino's influence arguably only remains in any clear sense for the four stated genes out of the eight genes analysed above. It should also be noted that the level of *B4* apparently steadily increases over time (fig.24) and the level of the internal *B4* control in fig.25 occasionally varies. This is particularly the case for sample C1C of the *HDAC* gel being possibly over-represented compared to the other cytoplasmic lanes. It is not thought the supposed experimental errors behind these slight deviations distorts the above interpretation however.

Therefore it is thought that a reduction in *Xp54* brought about by the antisense morpholino somehow leads to a stabilisation, at the stated times,

of the *mos*, *rpS1* and *histone H4* messages and leads to a destabilisation of the *FRGY1* message. It is interesting to note that overexpressing T7-Xp54 (figs.20 and 21) resulted in apparent destabilisation of *histone H4* message and *rpS1* and possibly stabilised *FRGY1* mRNA, the opposite effects of the presumed under-expression of Xp54 brought about by the antisense morpholino.

Thus the quantity of available Xp54 protein may affect the mRNAs for certain genes more than others as well as seeming to affect different mRNAs in contrary ways.

To attempt to counteract the apparent effect of the *Xp54* morpholino in reducing Xp54 expression in the oocyte, oocytes were injected with T7-tagged *Xp54* DNA in the nucleus or not injected, followed by injection of the *Xp54* antisense morpholino or the control morpholino in the cytoplasm. RNA was then extracted after 24 hours from 30 of each set of oocytes. RT-PCR was performed using primers for *rpS1* and *histone B4* to test for the levels of their mRNAs and the differences in these levels that may exist between oocytes T7-*Xp54* injected and oocytes injected with both morpholino and T7-tagged *Xp54* DNA. Thus another attempt was undertaken to further demonstrate by RT-PCR mRNA amplification that

the *Xp54* morpholino's apparent reduction of *Xp54* expression in the oocyte was influencing the net stability of mRNAs. The results are shown in figure 26.

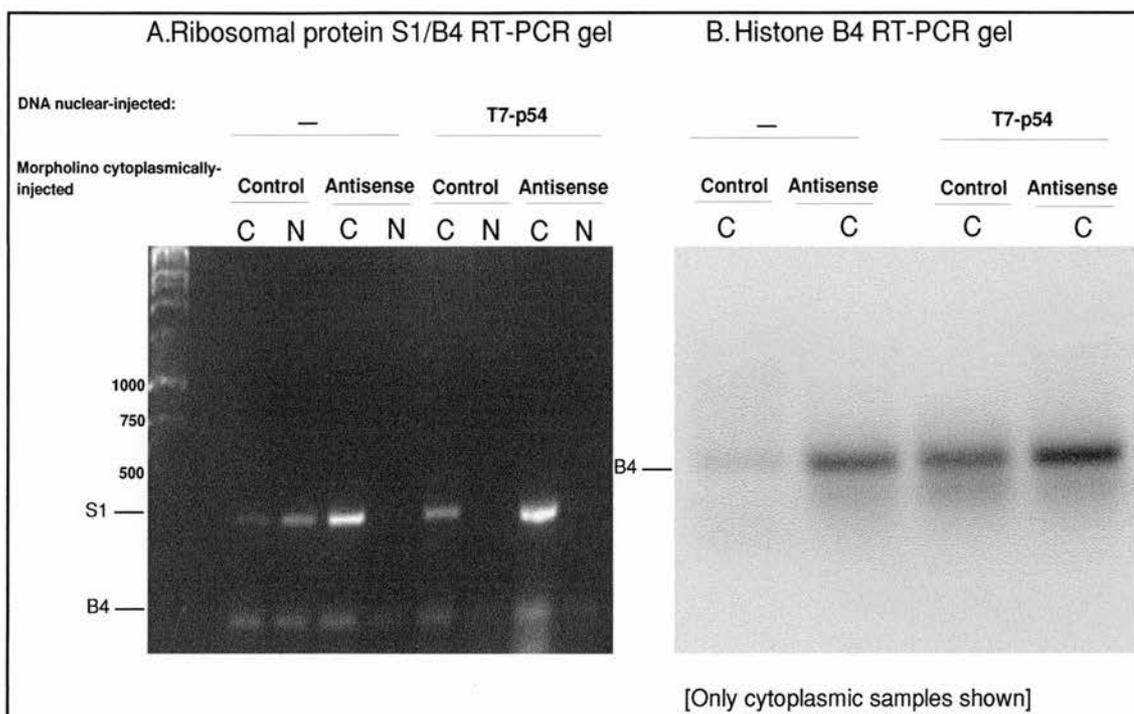


Figure 26: Exogenous Xp54 expression does not counteract the stabilisation of *rpS1* mRNA by the antisense morpholino.

RT-PCR reactions were performed on RNA extracted from the nuclei and/or cytoplasm of oocytes injected 24 hours previously with T7-*Xp54* DNA in the nucleus and/or anti-*Xp54* antisense morpholino in the cytoplasm. (a) Agarose gel for *rpS1* (and *histone B4* internal control); lower visible size-marker positions and positions of *rpS1* bands (367bp) and *histone B4* bands (150bp) indicated. (b) Agarose gel for *histone B4*. Position of *B4* band indicated.

Abbreviations: Control: injected with control-morpholino; Antisense: injected with the antisense *Xp54* morpholino only; T7-p54: injected with T7-*Xp54* DNA; —: not injected with T7-*Xp54* DNA.

The gel for *rpS1* (fig.26a) confirms the apparent stabilisation brought about by the antisense morpholino's injection for cytoplasmic *rpS1* mRNA as seen also over 24 hours and 48 hours in figure 25. The apparent (yet smaller) increase in nuclear *rpS1* mRNA brought about by the antisense morpholino over 24 hours as witnessed in fig.25 is not seen in fig.26 however. Looking at the internal *B4* control reveals a possible under-representation of *B4* mRNA in the nuclear antisense-only injected sample relative to the nuclear control-only injected sample. Thus experimental error in reaction tube or gel loading may have caused the nuclear uninjected-antisense band to be spuriously low but it is not known if the nuclear *rpS1* band would be as high let alone higher than the control-injected nuclear *rpS1* band in the absence of such error.

Combining the oocyte injections of T7-*Xp54* DNA and the antisense or control morpholino seems to significantly enhance the presence of the *rpS1* in the cytoplasmic samples particularly for the combined T7-*Xp54*/antisense injection although bands in the nuclear samples are not visible for *rpS1*. Also the combined injections seem to affect the *histone B4* mRNA presence relative to the T7-*Xp54* non-injected. The most notable change is the increase in cytoplasmic *rpS1* mRNA level for the T7-*Xp54*/antisense sample. The T7-*Xp54*/control morpholino injection cytoplasmic sample

shows a lower B4 intensity relative to the uninjected-control cytoplasmic sample and thus may represent some destabilisation of *histone B4* mRNA by overexpression of Xp54 that was not clear in figure 20.

In the case of the T7-p54/control cytoplasmic sample (fig.26) the exogenous Xp54 expression may have enhanced the nuclear export of rpS1 mRNA as suggested by figure 20 and the combination of this enhanced export with the presumed cytoplasmic stabilisation brought by the antisense morpholino may account for the largest rpS1 mRNA signal in the T7-p54/antisense cytoplasmic lane of the gel. This may also be the case for the *histone B4* mRNA although figure 20 did not suggest overexpression of Xp54 caused enhancement of *B4* export nor did figure 24 suggest clearly that the antisense morpholino stabilised *B4* mRNA. Thus the exogenous Xp54 expression may help to explain the absence of a visible band for rpS1 in the nuclear T7-p54/antisense and T7-p54/control samples although this would not account at all for the low nuclear signal in the uninjected-antisense lane.

In order to confirm the variations seen in the total mRNA levels of the *histone B4* used as an internal control in fig.26(a), reactions were subsequently set up to amplify *histone B4* mRNA alone and the results are shown in figure 26(b). An RNA gel run after pooling nuclear and

cytoplasmic samples (not shown) indicated that uninjected-control RNA was under-represented relative to other samples and had probably experienced some degradation which would account for the very low signal for the corresponding band in fig.26(b). However it can be seen that the combination of injected T7-*Xp54* and the antisense morpholino has apparently increased the stability of *B4* mRNA. Therefore it seems there might be a stabilising effect on *B4* mRNA by reducing *Xp54* expression which was not clear in figure 24 and combining of injections did not clearly negate the influence of each other but this may well be related to the fact that *rpS1* or *histone B4* did not show the more clear indication of *Xp54* overexpression-mediated mRNA destabilisation seen for a gene such as *rpL1* (fig.20).

In summary then it seems that in the presence of *Xp54* overexpression, the antisense morpholino is still able to stabilise *rpS1* mRNA and apparently stabilise *histone B4* at least at 24 hours (not clear in fig.24 due to lack of 24 hours control sample). This underlines the differential influence that *Xp54* apparently exerts on the mRNAs of different genes.

Injection of the *Xp54* antisense morpholino into progesterone-treated oocytes increases the stability of those mRNAs translated only during maturation

As stated in Introduction, certain mRNAs are stored as mRNP untranslated until after maturation or during embryogenesis. Therefore the apparent influence of *Xp54* on mRNAs might vary further according to the point in time within the oocyte's development. Consequently, stage 6 oocytes were cytoplasmically injected with the antisense morpholino or the control morpholino or left uninjected and after overnight incubation at room temperature (approximately 15-16 hours) maturation was induced by incubation in 300 µg/ml of progesterone. 50 oocytes were collected from each of the injected sets of oocytes and RNA extracted as described in Methods and Materials. Also the rate at which a group of 50 oocytes from each set of injected oocytes and from uninjected oocytes attained maturation was noted as indicated by the visible change of germinal vesicle breakdown (GVBD) appearing on the nuclear pole — the formation of a white spot. The kinetics of GVBD, *i.e.* the percentage of oocytes mature over the recorded period, is shown in figure 27.

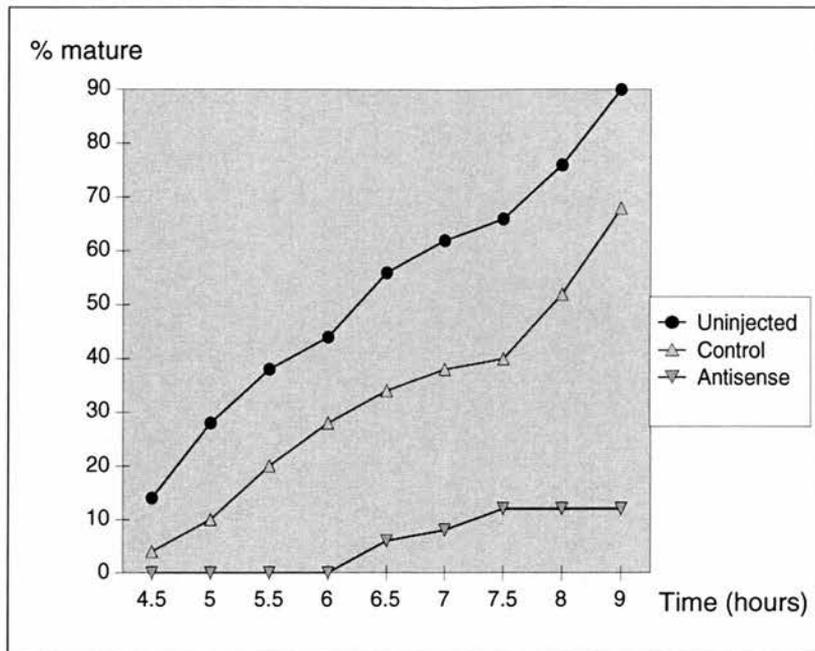


Figure 27: The antisense morpholino delays and inhibits oocyte maturation.

Graph shows percentage of oocytes maturing from pools of 50 that were either not injected, injected with the control morpholino or injected with the antisense morpholino. Maturation was recorded for 9 hours following addition of progesterone to the oocyte buffer. No set of oocytes reached 100% maturation over the recorded period.

The antisense morpholino clearly seems to both delay the onset of maturation and inhibit the process for at least 9 hours. Specifically, maturation only becomes noticeable in the antisense-injected pool after approximately 6.5 hours compared to at latest 4.5 hours in the non-injected and control-injected oocyte pools. This inhibition in maturation remains only a delay for a small percentage of antisense-injected oocytes even after 9 hours with less than 15% progressing to GVBD. Percentage maturation in the oocyte pool progresses rapidly after around 7.5 hours in the control- and non-injected oocytes but remains stationary in the antisense-injected oocytes.

The progress of maturation in the control-injected oocyte pool follows the pattern of the uninjected pool quite closely but a slight retardation in the rate of maturation is visible suggesting some maturation-inhibitory effect of the control morpholino. Unless this represents an unintended side-effect of the control morpholino such as targeting an unspecified mRNA then the injection process could have a mild inhibitory effect on GVBD.

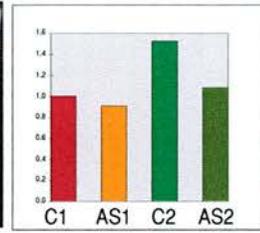
RT-PCR reactions were then undertaken using primers to amplify a range mRNAs with the aim again to obtain a reflection of their respective levels under the different experimental conditions — control injected and antisense treated — following exposure of oocytes to maturation-inducing

conditions. The agarose gels for these genes showing the products of the reactions along with histograms representing the densitometry performed on the gel bands are presented in figure 28.

mRNA levels at 3 hours and 9 hours following progesterone-induced oocyte maturation in antisense morpholino-injected oocytes

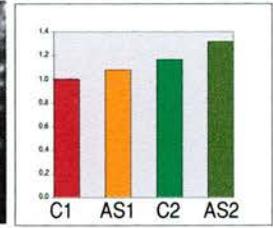
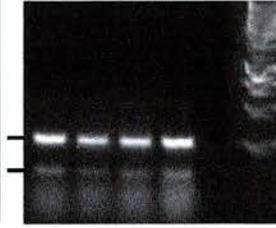
Tcf3/B4

C1 AS1 C2 AS2



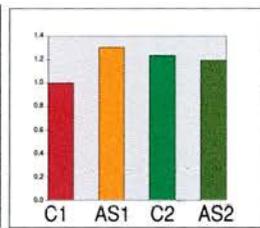
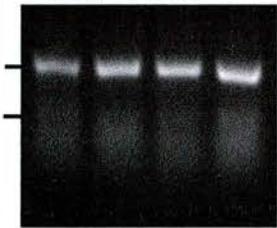
Histone H4/B4

C1 AS1 C2 AS2



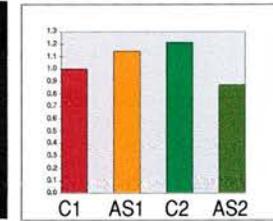
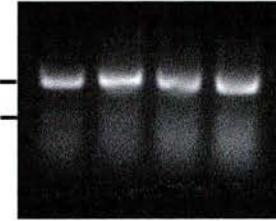
Beta tubulin/B4

C1 AS1 C2 AS2



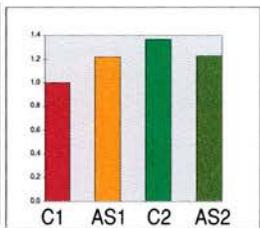
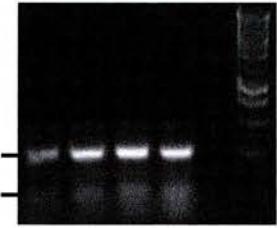
FRGY1/B4

C1 AS1 C2 AS2



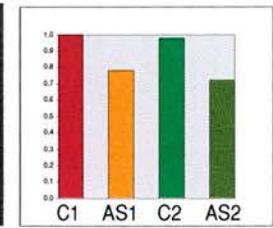
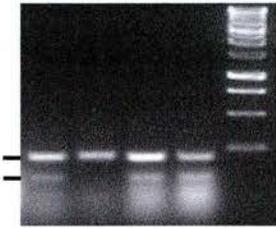
Histone Deacetylase/B4

C1 AS1 C2 AS2



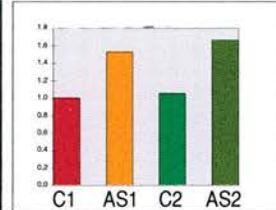
Ribosomal protein L1/B4

C1 AS1 C2 AS2



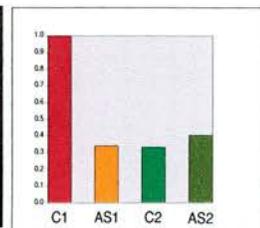
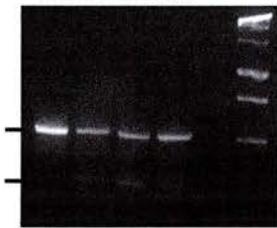
Histone B4

C1 AS1 C2 AS2



Mos/B4

C1 AS1 C2 AS2



Cyclin B1/B4

C1 AS1 C2 AS2

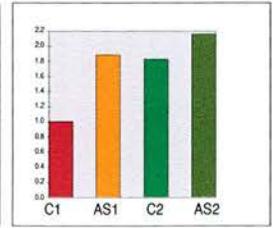
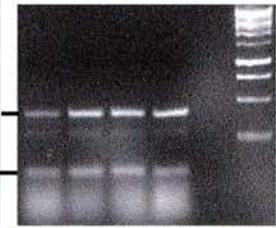


Figure 28: Injection of the *Xp54* antisense morpholino into progesterone-treated oocytes increases the stability of mRNAs only translated following maturation onset.

RT-PCR reactions were performed on RNA extracted from oocytes injected with either the control or antisense morpholino and exposed 12 hours later to 300µg/ml of progesterone. Extractions were made at 3 hours and 9 hours post-progesterone treatment. Agarose gels are shown for the mRNAs amplified along with histograms representing densitometry done on the gene's bands which is relative to a normalized value of 1 given to the C1 sample. Primers for *histone B4* were also included as an internal control for all reactions (the lack of a visible band in C1 and AS1 of the *mos* reaction and in AS1 of the *rpL1* reaction is noted).

Black bands indicate the target RT-PCR products, the lower band indicating *histone B4* in all cases.

Abbreviations: C1: control-injected sample at 3 hours; AS1: antisense-injected sample at 3 hours; C2: control-injected sample at 9 hours; AS2: antisense-injected sample at 9 hours; *tcf3*: transcription factor 3.

Figure 28 is arranged so that the results for genes known to be translated before maturation are in the upper part of the diagram (with the exception of *FRGY1* which is activated during embryogenesis) and those which are activated following induction of maturation in the lower part of the diagram (*histone B4*, *mos* and *cyclin B1*). The most obvious common property of the results which coincides with this distinction is the apparent increase in mRNA stability following 9 hours progesterone exposure and antisense injection seen in those genes which are translated only following maturation induction. In contrast, most of the remaining genes show an apparent decrease in mRNA stability brought about by antisense injection at this time. Given the maturation kinetics presented in fig.27 showing that before 4.5 hours none of the antisense-injected oocytes had matured and probably only a very small proportion of uninjected and control-injected oocytes had matured then it is probable that any notable changes in mRNA levels associated with the maturation process would only have occurred at sometime past the three hour time interval analysed in figure 28.

Furthermore at 9 hours there is a clear distinction between control-injected or uninjected oocytes and antisense-injected oocytes with regards to the proportion of mature oocytes. Therefore differences between mRNA levels at 9 hours would presumably be the most indicative of any influence of

maturation.

Thus the mRNA levels at 9 hours post-progesterone treatment in oocytes injected with the anti-*Xp54* morpholino are lower than under control conditions for the following genes: *transcription factor 3*, *beta-tubulin*, *histone deacetylase*, *FRGY1* and *ribosomal protein L1*. Consequently it can be postulated that a reduction of Xp54 protein levels during the maturation process leads to a lowering of the mRNA quantities for these genes by some means. Any differences in the mRNA levels in the first (3 hour) antisense-injected oocyte RNA samples relative to the control are thought to be less associated with the maturation process. Similarly the antisense morpholino-induced reduction in Xp54 is thought to be responsible for bringing about an increase in the mRNA levels for *mos*, *histone B4*, *histone H4* and *cyclin B1*.

Also by looking at figs.24/25 and fig.28 it can be seen that the influence of the antisense morpholino is apparently conserved before and after maturation induction at the lattermost time point (48 hours and 9 hours respectively) for *histone H4* and *mos* (both stabilised) and *FRGY1* (destabilised).

Neither *histone B4* or *cyclin B1* clearly showed any effect of the antisense *Xp54* morpholino at 48 hours following its injection in immature oocytes

(fig.24) but after maturation it seems clearer that the mRNAs of both genes were apparently stabilised by it (fig.28). In contrast, the mRNA for *rpL1* which also did not show a clear antisense-induced effect in immature oocytes was apparently destabilised by the antisense *Xp54* morpholino after maturation. Finally *HDAC* mRNA, which may have been stabilised by the *Xp54* antisense morpholino before maturation (fig.25) appears to have been destabilised following maturation induction (fig.28).

In view of the apparent influence of the anti-*Xp54* morpholino on *histone B4* mRNA levels post-maturation induction, the mRNA levels of certain samples in fig.28 may be under-represented (through loading error or interference between primers for example a primer of one gene spuriously priming a reaction from the transcript of the other gene thus detracting from its final intensity). Consequently the AS1 samples of *histone H4* and *rpL1* might be affected in this way although these allowances would not be expected to alter the conclusions arising from this analysis significantly. In contrast, possible under-representation in the AS2 sample of *pcf3* might weaken the case slightly for an antisense-induced destabilisation of its mRNA. Also in the C1 sample of *mos* with the absence of a clearly visible *histone B4* control, loading errors need to be taken into account in any conclusion.

Therefore it seems that for certain genes the influence of Xp54 on its mRNA alters before and after oocyte maturation induction. For some genes, such as *HDAC*, on whose mRNA Xp54 may have had a net destabilising effect before maturation, this effect is lessened or becomes stabilising. For other genes, for which there are more examples in those analysed above, such as *rpL1*, *histone B4* and *cyclin B1* where there seems to be less discernible influence by Xp54 in immature oocytes, an influence is more strongly suggested after maturation induction. For other genes such as *histone H4* and *FRGY1*, the net effect of Xp54 on their mRNA's stability may be the same in immature and maturing oocytes.

Despite this variability in apparent Xp54 influence it nevertheless remains striking that for the set of genes analysed here, such influence appears to be overall a stabilising one on mRNAs from genes translated after maturation induction and more commonly a destabilising one for those genes which have been translating in immature oocytes.

An antisense morpholino directed against the mRNA masking protein FRGY2 affects the stability of mRNAs depending on translational status

The apparently differential effect of reduced Xp54 levels on mRNA stability which, as stated, may commonly relate to the particular mRNA's participation in translation suggests that by inhibiting the production of other major mRNP proteins with which the mRNA associates, the stability of the mRNA may again be affected.

Bearing in mind the proposed 'maskosome' interaction of the Y-box masking protein FRGY2 with Xp54 within mRNP particles (Sommerville, 1999), stage 4 oocytes were cytoplasmically injected with an antisense morpholino designed to target *FRGY2* mRNA in order to reduce the presence of FRGY2 within the oocyte. Oocytes were injected with a concentration of 1nm/μl of anti-*FRGY2* morpholino (sequence: 5' gctcctgggcttccgcctcactcat 3') or a control morpholino of the same random sequence as used previously. 25 oocytes were then collected from both the uninjected and injected sets of oocytes at 24 hours and 48 hours post-injection during which time the oocytes remained at room temperature. Oocytes were then RNA extracted as described in Methods and Materials

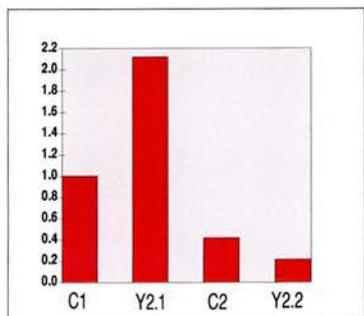
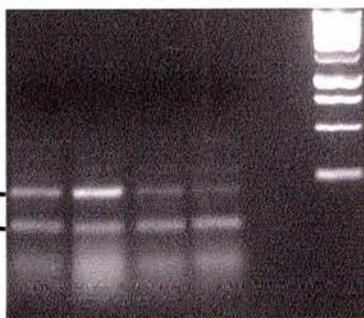
giving four RNA samples: two control-injected and two anti-*FRGY2* morpholino injected,

RT-PCR reactions were then undertaken using primers for a range of genes to gain a reflection of mRNA levels for these genes under the experimental conditions imposed — control and inhibition of *FRGY2* translation. The agarose gels showing the reaction products and histograms representing the densitometry performed on the gels are shown in figure 29.

mRNA levels following oocyte injection with antisense FRGY2 morpholino

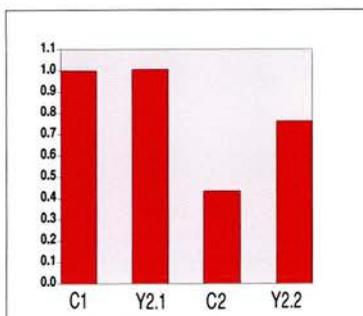
Ribosomal protein L1/B4

C1 Y2.1 C2 Y2.2



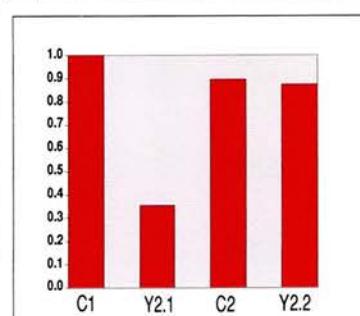
Tcf3/B4

C1 Y2.1 C2 Y2.2



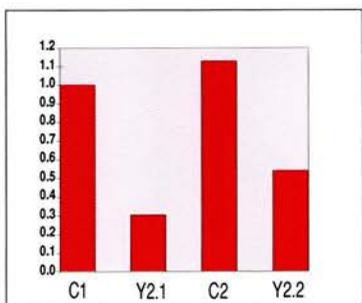
FRGY1/B4

C1 Y2.1 C2 Y2.2



Mos/B4

C1 Y2.1 C2 Y2.2



Cyclin B1/B4

C1 Y2.1 C2 Y2.2

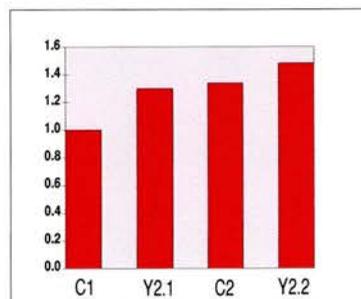
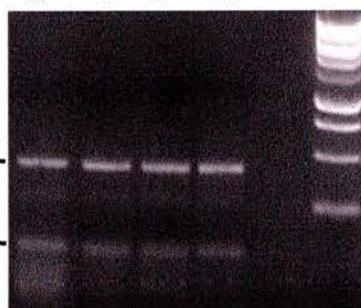


Figure 29: Injection of a *FRGY2* antisense morpholino into oocytes differentially affects the stability of mRNAs.

RT-PCR reactions were performed on RNA extracted from stage 4 oocytes injected with the control or the anti-*FRGY2* antisense morpholino. Extractions were made at 24 hours and 48 hours post-injection giving 4 samples. Agarose gels showing products of reactions for each gene analysed and histograms representing densitometry of the gene's bands are displayed. Molecular size markers are in the rightmost position of each gel and the lower four sizes are 1000, 750, 500 and 250bp. Densitometry for each gene is normalized to a value of 1 given to the C1 sample. Primers for *histone B4* were also included as an internal control for all reactions. *B4* bands are the lowest bands, where visible, in all cases.

Black bands indicate the target RT-PCR products, the lower band indicating *histone B4* in all cases.

Abbreviations: C1: control-injected at 24 hours; Y2.1: antisense-injected at 24 hours; C2: control-injected at 48 hours; Y2.2: antisense-injected at 48 hours.

The variable pattern of densitometry between each gene analysis indicates a varying influence of the anti-*FRGY2* antisense morpholino according to the mRNA in question as was the apparent nature of the anti-*Xp54* antisense morpholino. However, the effects of the anti-*FRGY2* morpholino are arguably clearer and noticeable than some of the results for the anti-*Xp54* morpholino (figs.24 and 25) indicated. Whilst a gene such as *FRGY1* indicates that its message may have been destabilised indirectly by the anti-*FRGY2* morpholino (fig.29) as the anti-*Xp54* morpholino did (fig.24), *rpL1* and *cyclin B1* indicate a more definite anti-*FRGY2* morpholino influence (fig.29) than was implied by the anti-*Xp54* morpholino. *Mos* mRNA, which showed stabilisation by the anti-*Xp54* morpholino at 48 hours post-morpholino treatment, is affected in the reverse way by the anti-*FRGY2* morpholino with consistent destabilisation indicated over both time points.

The *histone B4* control is reasonably consistent in intensity between the lanes of each gene analysis with the exception of the C1 and Y2.2 samples of the *pcf3* reactions which vary more suggesting some interference between the primers for *pcf3* and *histone B4* mRNA amplification, for example one primer being able to bind and prime a reaction on the non-target sequence detracting from the other bands intensity. Any

densitometry errors arising through loading error however would be expected to widen the differences presented in the histogram for *pcf3*. The *histone B4* control is not clear in the *FRGY1* reaction suggesting some inhibition of the reaction or its presence at a level not detected by the level of sensitivity used for the gel photography because of a large quantity of *FRGY1* message outcompeting the *histone B4* message for reaction ribonucleotides in the RT-PCR process.

The apparent stabilisation of some messages by the *FRGY2* antisense injection and destabilisation of others may relate to the nature of the influence of *FRGY2* on a particular mRNA assuming an association or interaction exists. With the maskosome hypothesis in mind, involving *FRGY2* translationally repressing mRNA, an inhibition of *FRGY2* translation by the antisense morpholino would not be the most immediate logical cause of a stabilisation of another message as is apparent for *cyclin B1*, *rpL1* and possibly *pcf3*. The reduction in cellular *FRGY2* might be thought to lead to less masking and more subsequent translation and degradation of an mRNA as possible for *mos* and *FRGY1*. On the other hand a role for *FRGY2* in promoting translation as its somatic homologue, *FRGY1*, is thought to be capable of (Evdokimova and Ovchinnikov, 1999) may help to explain cases of likely *FRGY2* antisense morpholino-induced

stabilisation. In this way a reduction in the amount of available FRGY2 protein would lead to less translation and subsequent degradation of a message with which it normally associates perhaps in a quantity that is lower than that required to mask the message but which can enhance the translation rate. This promotion of translation could therefore underlie the apparent anti-*FRGY2* morpholino stabilisation of message seen with *rpLI* and *tcf3*. How a reduction in FRGY2 levels and presumably masking would result in enhanced stability of *cyclin B1* mRNA (a non-translated message) however would be thought to require an alternative explanation. A different regulation of FRGY2's interaction with mRNA or a different nature of interaction such as a greater quantity of FRGY2 involved may cause masking and an inhibition of translation and degradation that is counteracted by the antisense morpholino in the *mos* and *FRGY1* reactions.

The contrary results between the two time points for *rpLI* could be partially explained by what might be an intron-containing form of the *rpLI* band that has been amplified in the reactions (between the 250bp and 500bp size markers in fig.29) which is of higher intensity in the Y2.2 lane than in the C2 lane so possibly detracting from the main, intended *rpLI* band (240bp) which was subject to densitometric analysis. The intensities of the *rpLI*

bands in both lanes of the second time point are markedly less than in the first time point which, by comparing with the *histone B4* bands, seems to indicate a selective degradation of *rpL1* messages in both control-injected and morpholino-injected samples at this timepoint which in light of the notably lower quantity of *rpL1* mRNA in the 48 hours control sample of figure 24 may raise questions about the continuity of transcription of genes during oogenesis.

Xp54 inhibits and FRGY2 promotes general oocyte mRNA translation

In the experiments described above, the specific effects of the overexpression of Xp54 and the impaired expression of Xp54 or FRGY2 have been examined. That is, the changes in particular mRNA levels or tagged Xp54 levels apparently brought about as a result of the nature of the oocyte injections. Attention in this investigation was then focussed on the effects of reducing Xp54 and FRGY2 synthesis on protein levels in general, that is, their synthesis and the stability.

Accordingly sets of 50 stage 3 oocytes were injected separately with the control morpholino, the anti-*Xp54* morpholino or the anti-*FRGY2* morpholino. After 24 hours incubation at room temperature in 100µl OR2 buffer, 2µl of ³⁵S methionine was added to the buffer (30µCi in 100µl OR2)

along with 0.5 μ g/ml alpha-amanitin (to inhibit further transcription and therefore to aim to maximise the effect of the morpholino on the oocyte protein pool) and the oocytes incubated for a further 24 hours before protein was extracted by freon extraction as described in Methods and Materials. Additionally 3 oocytes from the 3 sets injected with each morpholino were taken at 6, 12 and 24 hours post label addition and washed in fresh OR2 buffer. These oocytes were then squashed onto filter paper and the proteins precipitated with trichloroacetic acid as described in Methods and Materials and the relative counts per minute were measured using a Geiger counter in order to determine the time course of radioactive sulphur incorporation into proteins and thus gain an idea of the relative rates of protein synthesis in each set of oocytes. The incorporation kinetics are represented by figure 30.

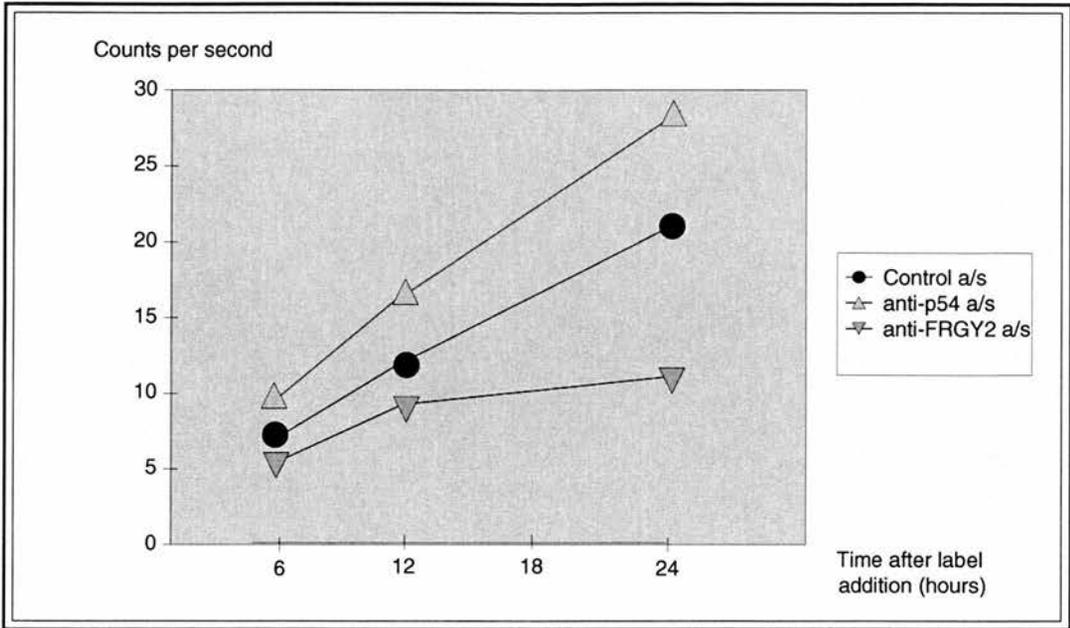


Figure 30: ^{35}S methionine incorporation into proteins is enhanced by inhibition of Xp54 translation and reduced by inhibition of FRGY2 translation. Equal numbers of stage 3 oocytes injected with one of three morpholinos (anti-Xp54 antisense morpholino, anti-FRGY2 antisense morpholino or the control morpholino) were measured for radioactive ^{35}S methionine incorporation at three time-intervals after addition of label to the oocyte buffer, itself 24 hours after morpholino injection.

The graph indicates a consistently higher level of radioactivity incorporation (and therefore presumably of protein synthesis) in the anti-*Xp54* morpholino-injected oocytes than the control and also the anti-*FRGY2* morpholino-injected oocytes that is consistently lower than the control level. However until approximately 12 hours post label addition the rates of incorporation appear roughly the same though the levels would have presumably been attained by varying rates before the 6 hour interval. After 12 hours the rates increase roughly proportionally to the respective degrees of incorporation at that point. That is, the anti-*Xp54* morpholino-injected oocytes show the greatest increase in rate of incorporation followed by the control morpholino-injected oocytes then the anti-*FRGY2* morpholino-injected oocytes. Thus a significant enhancement or reduction in total protein synthesis is thought to be brought about and sustained for at least 48 hours following the injection into the oocyte of the anti-*Xp54* or anti-*FRGY2* morpholino respectively. It is therefore assumed that the inhibition of *Xp54* or *FRGY2* translation is responsible for this respective enhancement or reduction.

The levels of total protein synthesis were then examined by separation of the 3 extracted oocyte samples by SDS-PAGE and autoradiography to visualize all (or the presumed majority of) proteins synthesised following

addition of the radiolabel, at total of 48 hours after morpholino injection.

This separation is presented in figure 31.

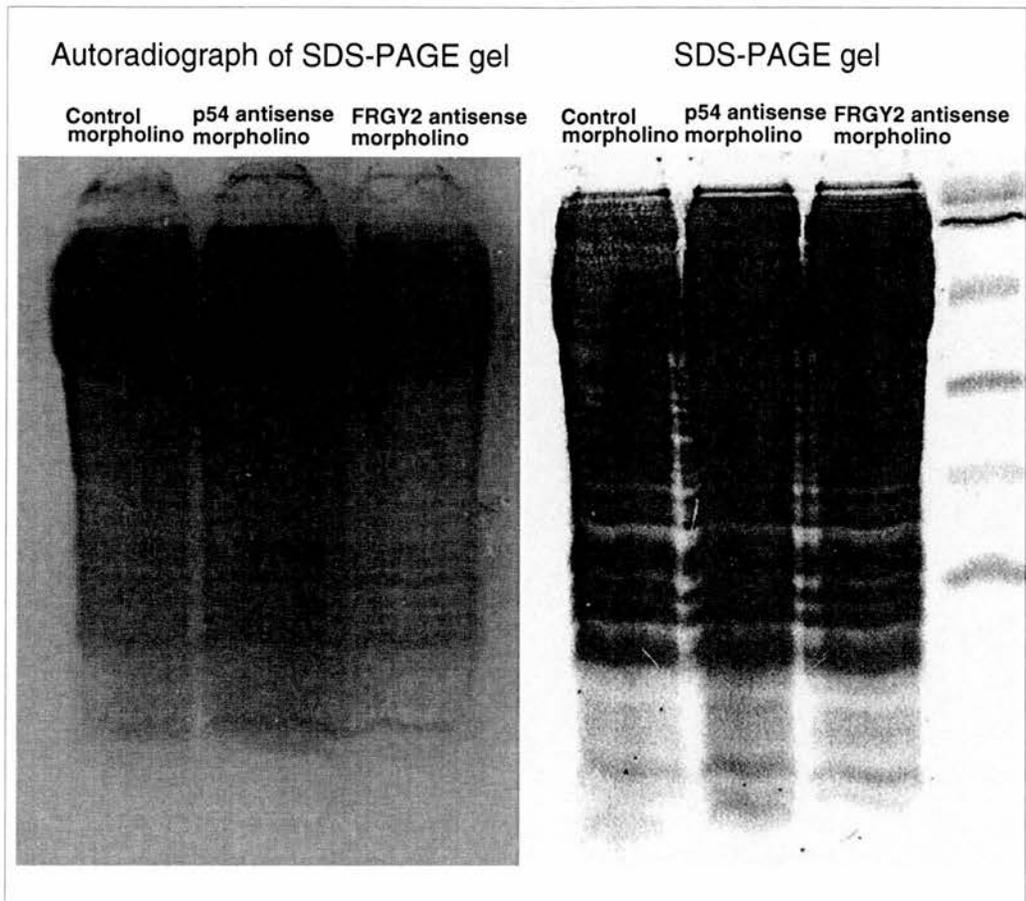


Figure 31: Inhibition of *Xp54* translation enhances total protein synthesis and inhibition of *FRGY2* translation reduces total protein synthesis.

Equal numbers of stage 3 oocytes injected with one of three morpholinos (anti-*Xp54* antisense morpholino, anti-*FRGY2* antisense morpholino or the control morpholino) were incubated for 24 hours at room temperature and then treated with ^{35}S methionine radiolabel and alpha-amanitin before a further incubation of 24 hours. Protein was then extracted from each set of oocytes and equal quantities subject to SDS-PAGE. The resulting gel and the results of autoradiography of the gel are shown. Samples loaded are, (from left in each photograph), control morpholino-injected oocytes, anti-*Xp54* morpholino-injected oocytes and anti-*FRGY2* morpholino-injected oocytes.

The autoradiograph gives a visualization of those proteins from the total extracted proteins separated from each sample on the SDS-PAGE gel that were synthesised after radiolabel addition. That is, those proteins synthesised within the oocyte in the 24 hours before protein extraction whilst the oocytes were still under the presumed influence of the morpholinos. *Xp54*-antisense morpholino-injected oocytes show a noticeably higher amount of proteins than both the control-injected and anti-*FRGY2* morpholino injected oocytes. This difference is noticeable across virtually all of the size range of proteins visible on the autoradiograph. A similarly visible reduction in protein synthesis is indicated for the anti-*FRGY2* morpholino injected oocytes which has the lowest quantity of protein present of all the three samples. On the SDS-PAGE gel, a higher quantity of protein is visible at the lowest molecular weights for the anti-*Xp54* morpholino-injected oocytes and a slightly higher total amount of protein compared to the control morpholino-injected oocytes. This suggests that the presumed enhancement of protein synthesis induced by the anti-*Xp54* morpholino is sufficient to make its influence visible on the total protein pool which includes those proteins which were synthesised prior to morpholino injection and persist at 48 hours post-injection. The impact of the anti-*FRGY2* morpholino is apparently less

marked however.

Therefore as stated for fig.30 the differences between the results of the three separate samples displayed in fig.31 are thought to be a result of the reduction in *Xp54* or *FRGY2* mRNA translation caused by the respective morpholino's influence. Consequently, given the association of both proteins with maternal mRNA in the oocyte, the morpholino-induced reduction in *Xp54* protein could be leading to a stabilisation and/or enhanced translation of most oocyte mRNAs and the reduced levels of *FRGY2* protein brought about by the anti-*FRGY2* morpholino could be leading to a destabilisation and/or reduced translation of oocyte mRNAs. It follows therefore that *Xp54* and *FRGY2* might have general functions in mRNA destabilisation (or alternatively inhibition of general translation by other means) and stabilisation (or promotion of general translation by other means) regardless of the specific effects on the individual genes analysed by RT-PCR above.

The possibility exists that the presumed enhancement and reduction in total protein synthesis brought about by the respective morpholinos represent effects on the stability of oocyte proteins, somehow achieved by a reduction in the two target proteins, *Xp54* and *FRGY2*. Therefore, in order to gain an ideally more accurate view of new protein synthesis 24 hours

after morpholino injection, the relative amount of total protein synthesis in oocytes injected with the anti-*Xp54* or anti-*FRGY2* morpholinos used above was also investigated by extracting polyA(+) material. Three sets of stage 3 oocytes (anti-*Xp54* injected, anti-*FRGY2* injected or uninjected) were incubated for 12 hours at room temperature before addition of 1µl of ³⁵S methionine (15µCi in 100µl OR2) to the buffer and incubation for a further 12 hours before polyA(+) extraction as described in Methods and Materials. Poly(A)+ material includes nascent polypeptides emerging from polysomes and so should provide a more selective method, that is to obtain a visualization of newly synthesised proteins. The three resulting protein samples were separated by SDS-PAGE and the SDS-PAGE gel used to obtain an image of proteins synthesised after radiolabel addition to the oocytes by autoradiography (figure 32).

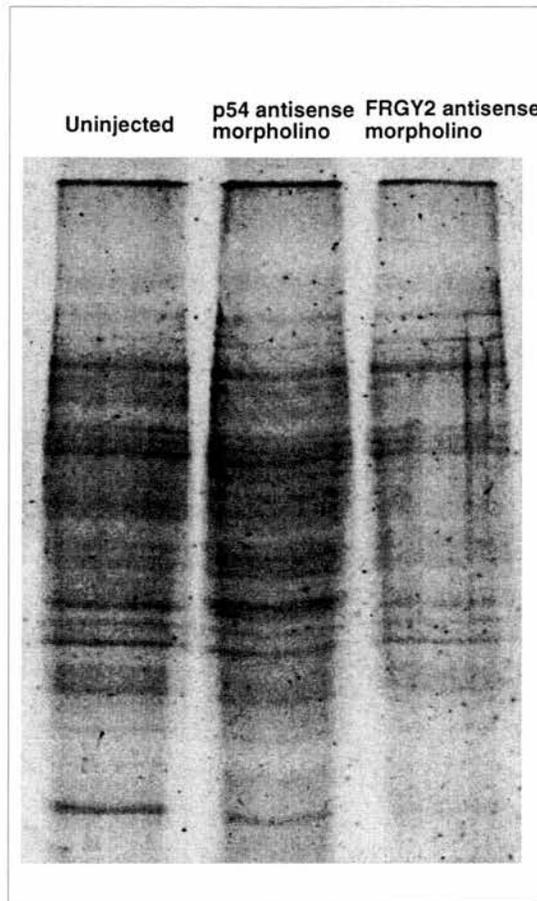


Figure 32: New protein synthesis is enhanced by inhibition of *Xp54* translation and is reduced by inhibition of *FRGY2* translation.

Stage 3 oocytes were injected with the anti-*Xp54* morpholino, the anti-*FRGY2* morpholino or left uninjected and incubated at room temperature for 12 hours before addition of ^{35}S methionine radiolabel to oocyte buffer and incubation for a further 12 hours. Poly(A)+ material was then extracted and separated by SDS-PAGE and autoradiography undertaken. The autoradiograph is shown with samples (from left): uninjected oocytes, anti-*Xp54* morpholino-injected oocytes and anti-*FRGY2* morpholino-injected oocytes.

As seen in fig.31, and in the methionine incorporation kinetics, the pattern of the quantity differences between the anti-*Xp54* injected, anti-*FRGY2* injected and control/uninjected samples is conserved for the poly(A)+ material. Figure 32 shows the greatest amount of material in the anti-*Xp54* morpholino-injected oocytes across the middle size-range of proteins which encompasses the most prominent proteins present. The uninjected oocytes show a lower quantity of poly(A)+ material across this size-range and the anti-*FRGY2* morpholino-injected oocytes show the least.

Thus the presumed reduction in oocyte *Xp54* protein caused by morpholino treatment is thought to lead to an increase in the new synthesis of a wide range of the most prevalent oocyte proteins, indicated to be occurring for at least 24 hours post-morpholino injection by fig.32. The influence of the anti-*FRGY2* morpholino is, similarly, at least as long, with a reduction in new synthesis of most prevalent proteins in the oocyte. Thus it is thought most likely that the effects of *Xp54* and *FRGY2* are in generally inhibiting translation and promoting translation by some means (for example by effects on mRNA stability) as opposed to affecting general protein stability.

Interaction of Xp54 and RAP55 within mRNP particles

A possible candidate for an Xp54 cofactor as stated in Introduction, is RAP55 (Lieb *et al.*, 1998) that could represent the major *Xenopus* mRNP particle component p52/RNP2 (Darnborough and Ford, 1981). Unpublished results (J.Sommerville, pers. comm.) of peptide sequencing on the 52KDa protein extracted from SDS-PAGE gels (peptide sequencing by G.D. Kemp, St.Andrews) indicate regions of identity to the peptide sequence of a human protein (UNIPROT accession no. Q9BX40) in publicly accessible databases that itself is identified by its homology to RAP55 (Q9YH12) originally identified by Lieb *et al.* (1998). Also, access to the publicly available protein databanks reveal an amino acid mis-count for RAP55 in Lieb's *et al.* (1998) paper of 11 too many which means the correct predicted molecular mass is 51.9KDa rather than 55KDa (however, for simplicity the protein will be referred to as RAP55 or Q9YH12). Thus there are indications that p52/RNP2 represents the orthologue to *Pleurodeles* RAP55.

Unpublished results (fig.33) reveal the presence of Xp54 and p52 in the same protein complex following UV-crosslinking treatment and immunoblotting with antisera raised against *Xenopus* Xp54 or against extracted p52 protein. This convergence of the proteins does not appear to

depend upon mRNA whose absence does not decrease the intensity of the complex and may even increase it slightly. The complex is at a much higher molecular weight than the monomeric forms of Xp54 and p52 as shown in figure 33.

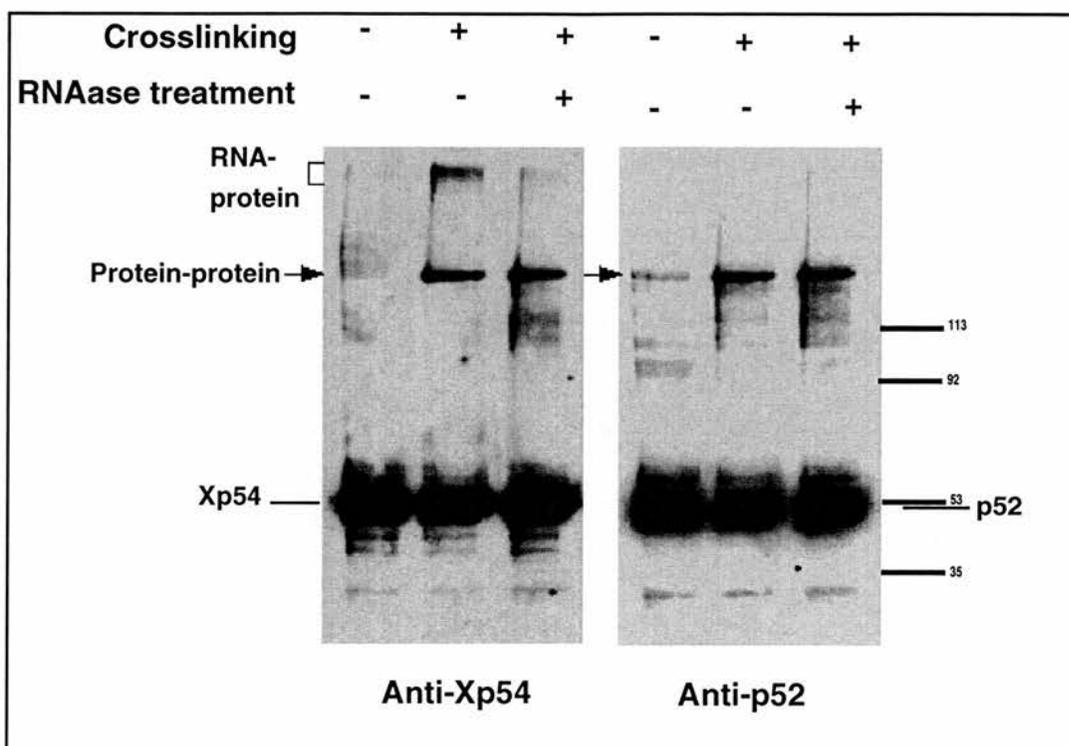


Figure 33: Xp54 and p52 form coincident complexes following UV-crosslinking treatment.

Immunoblots show poly(A)+ material-associated protein extracted from stage 1 oocytes by oligo-d(T) affinity beads that was either untreated, crosslinked by UV-treatment at 254nm for 15 minutes on ice or UV-treated and then RNAase treated (1µg/ml) for 30 minutes at 37°C. Treatments applied or not are indicated by '+' or '-' symbols.

Suspected RNA-protein and protein-protein interactions are indicated based on the type of treatment received. A high quantity of Xp54 and p52 in the collected oocytes is thought to have caused overloading of either respective protein on the gel.

Both immunoblots are aligned according to the same molecular size-marker calibration. Only the separating portion of the blotted gel is shown.

Work undertaken by Dr.D.Smillie (St.Andrews).

Therefore, as indicated by fig.33, there exists further evidence that Xp54 and p52 are in close proximity in the same cellular fraction in the oocyte. Experiments designed to better understand the nature of this protein's presence in mRNP particles thus should prove valuable to understanding the regulation of Xp54.

With this in mind, investigations were carried out to examine the nature of any interactions between Xp54 and RAP55. Stage 4 oocytes were injected with the T7-*Xp54* expression vector DNA and separately with the same vector and an expression vector carrying an initial cDNA candidate for the orthologue of *Pleurodeles* RAP55 in *Xenopus*. Another set of oocytes was left uninjected.

The candidate was identified from the expressed sequence tag (EST) databank of the EBI¹ as two *Xenopus* ESTs which showed similarity to the 5' and 3' ends of the cDNA for RAP55 (Q9YH12) of *Pleurodeles*. A clone was then ordered of an expression vector harbouring a *Xenopus* cDNA from which the ESTs had been derived (I.M.A.G.E Consortium², clone no.: 3405473). The expression vector consists of a pCMVsport6 plasmid with a CMV eukaryotic cell expression promoter. After 48 hours, 35 oocytes from each set injected were collected and protein extracted by freon extraction as

¹ European Bioinformatics Institute: www.ebi.ac.uk

² <http://image.llnl.gov>

described in Methods and Materials, following addition of 200µl mRNP binding buffer (as used for immunoprecipitation) for homogenisation. 40µl of each of the three samples was taken for UV-crosslinking to test for proximity of interacting proteins or UV-crosslinking and RNAase treatment (1mg/ml) to digest mRNA in the sample and test for any interactions involving mRNA. This gave a total of 9 samples as arranged below:

Treatment of extracted material	Expression vector(s) injected
Not crosslinked	Uninjected
	T7- <i>Xp54</i> injected
	T7- <i>Xp54/RAP55</i> candidate
Crosslinked	Uninjected
	T7- <i>Xp54</i> injected
	T7- <i>Xp54/RAP55</i> candidate
Crosslinked and RNAase treated	Uninjected
	T7- <i>Xp54</i> injected
	T7- <i>Xp54/RAP55</i> candidate

Of the nine samples, those injected with one or both vectors were immunoprecipitated with anti-T7 antibodies to extract T7-Xp54 containing mRNP particles. 10 μ l of each sample was then loaded giving the equivalent material of roughly 1.75 oocytes loaded per lane. Immunoblotting was then performed using anti-Xp54 antibodies. Thus a more specific level of selection for Xp54-containing structures was employed that was not used for those samples shown in figure 33, that of immunoprecipitation. This was intended to select those structures formed following expression of the injected T7-Xp54 DNA as opposed to all poly(A)+ mRNA-associated structures.

The immunoprecipitation of T7-Xp54 containing complexes reveals the presence of Xp54 in the same particle indicating assembly of both proteins into mRNP particles at some point following translation of T7-Xp54 mRNA. The immunoblot also shows however the presence of an Xp54- and T7-Xp54 containing complex at a position above the respective migrated distances for the two proteins (labelled). The less-defined nature and close proximity to the supposed monomeric forms of the proteins may indicate that it is a form or fraction of the total pool of Xp54/T7-Xp54 that becomes complexed with mRNA. The injection of the T7-Xp54 increases the quantity of this complex. Also this increased quantity seems more susceptible to RNAase action as can be seen by the lower intensity of the corresponding band in the XR lanes of the T7-Xp54 injected and T7-Xp54/RAP55(candidate)-injected samples relative to the O lanes. The anti-Xp54 appears to bind more strongly to the T7-Xp54 form of the protein in contrast to the results in fig.18 although this is likely to be due to the selection for T7 epitope-containing particles arising from immunoprecipitation.

The crosslinking procedure appears to cause a reduction in the Xp54 and, to a lesser extent, T7-Xp54 band intensity that is noticeable in both of the crosslinked T7-Xp54 injected and T7-Xp54/RAP55-injected samples relative to the non-crosslinked samples. This suggests close proximity to

some protein that either Xp54 form crosslinks to but is not identifiable on the immunoblot.

There are marginally lower T7-Xp54 band intensities seen in the T7-*Xp54/RAP55*-injected samples relative to the corresponding T7-*Xp54* injected samples which could indicate a direct or indirect Xp54-interaction, specifically with RAP55, that is too large to transfer from the SDS-PAGE gel resulting from the overexpression of the *RAP55* candidate that detracts from the monomeric T7-Xp54 band.

The T7-*Xp54/RAP55* DNA injections were then repeated and as before samples were taken for crosslinking, crosslinking and RNAase treatment or left untreated before immunoprecipitation with anti-T7 antibodies and immunoblotting with anti-RNP2 or anti-T7 antibodies. Thus a direct attempt was made to visualize if the vector expressing the RAP55 orthologue candidate was recognised by antibodies previously raised against p52. One variation that was taken between this run and the previous co-injection experiment was that RNAase treatment was performed before rather than after UV-crosslinking in the crosslinked/RNAase treated sample. The resulting immunoblots are shown in figure 35.

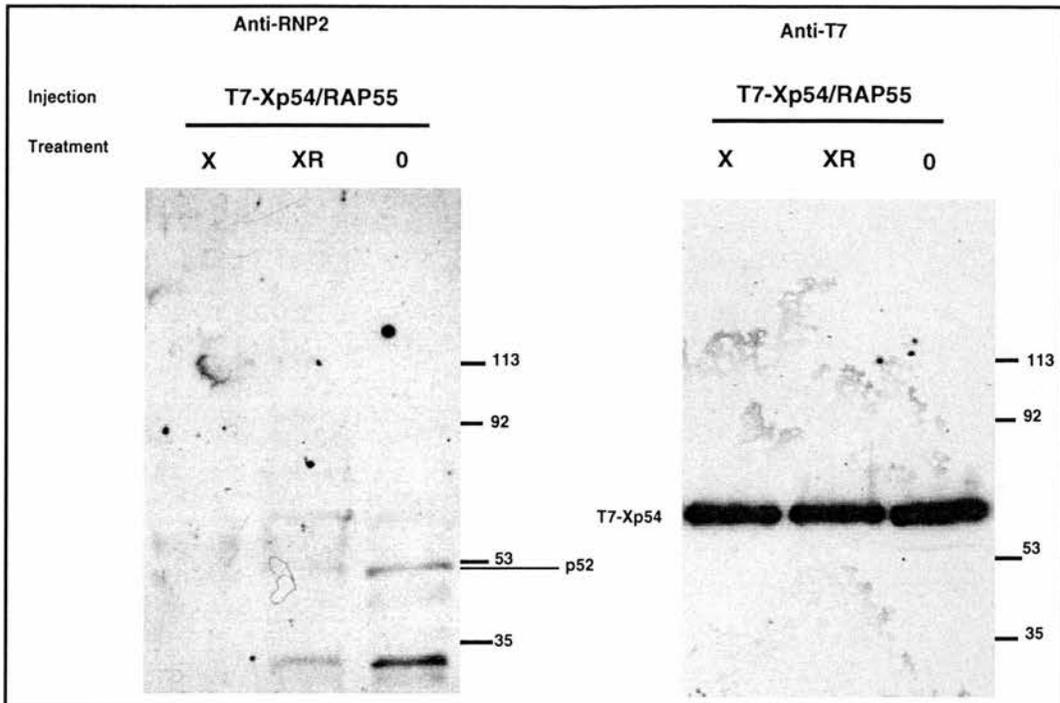


Figure 35: The presence of RNA promotes crosslinking of p52 to unidentified factors.

Protein was extracted from stage 4 oocytes injected 48 hours previously with T7-Xp54 expression vector and a *RAP55* candidate expression vector. Samples were then either left untreated, UV-crosslinked or RNAase treated then UV-crosslinked. Injected samples were then subject to immunoprecipitation to extract T7-Xp54 containing particles. All samples were then separated by SDS-PAGE and immunoblotted by anti-RNP2 antibodies then anti-T7 antibodies. Molecular size positions are indicated as are the positions of the target proteins, RAP55 being detected at a position corresponding to an apparent M_r of 52KDa.

Abbreviations: O: untreated; X: subject to UV-crosslinking; XR: subject to RNAase treatment then UV-crosslinking.

RNP2 is detected at a position of approximately 52KDa in the untreated sample as expected but disappears from detection when the sample is crosslinked yet re-appears albeit at a lower intensity when the sample is treated with RNAase prior to crosslinking. In conjunction with the anti-T7 immunoblot the result for the untreated sample confirms the presence — and thus incorporation into — of RNP2 in T7-Xp54 containing structures. Thus the presence of RNA facilitates crosslinking to one or more factors such that p52 becomes undetectable at the position associated with its presumed monomeric form after crosslinking and virtually undetectable in the presumed reduced presence of RNA brought about by RNAase treatment. This is in contrast to figure 34 in which Xp54 and T7-Xp54 are apparently not reduced to such an extent by crosslinking in quantity of their monomeric form in the presence or a reduced presence of RNA.

Neither figure 34 nor 35 clearly demonstrate a band which could be attributed to expression from the *RAP55* candidate expression vector. This is due to the lack of a visible band of higher molecular weight in the T7-Xp54/*RAP55* samples of fig.34 and the fact that the p52 visible in fig.35 could be endogenous protein that has complexed with T7-Xp54. Samples prepared in the same way as those in fig.35 but from oocytes injected either

with T7-*Xp54* DNA or the *RAP55* candidate expression vector alone then immunoblotted with anti-RNP2 would need to be compared to make the determination of expression from the latter vector.

It is not clear what p52 complexes to by crosslinking which is either too large to enter the SDS-PAGE gel (the whole gel indicates tightly packed bands around the well) or for some other reason is not revealed on the anti-RNP2 immunoblot in fig.35. Therefore, the presence of p52 in a p52- and *Xp54*-containing higher molecular weight band as seen in figure 33 after poly(A)+ material extraction in stage 1 oocytes is not observed following selection by immunoprecipitation of newly-formed T7-*Xp54* containing structures in stage 4 oocytes and immunoblotting with the same antibody for both extractions (fig.35).

In view of the uncertainty regarding the identity of p52 and the extent of any evolutionary relationship to *Pleurodeles* *RAP55*, it was decided to sequence the cDNA of clone IMAGE:3405473 together with another clone also identified from the databanks through EST similarity to the 5' of *RAP55* cDNA and perform alignments of their translated amino acid sequences. Thus it was hoped to identify the most likely cloned candidate for the *Xenopus* *RAP55* orthologue and to determine that if there was a p52 clone what would be the extent of its similarity to *RAP55* and the human

RAP55-like protein.

The details of the ESTs used to identify the *RAP55*-like cDNAs contained in IMAGE clones are shown in table 3.

Sequencing was performed as described in Methods and Materials. IMAGE 3405473, the original clone that was microinjected in figs.34 and 35, is hereinafter referred to as XRAPA and the second clone (IMAGE 3473035) is referred to as XRAPB.

The sequences generated from the IMAGE clones are provided in Appendix B together with a translation of the protein-coding sequence, the sequences of the sequencing primers and the position of the primers on the full-length cDNA and ESTs. Alignments of the translated sequences of XRAPA and XRAPB with each other, *RAP55* and the human *RAP55*-like protein (Q9BX40, which showed similarity to the extracted p52 peptide fragments as stated above) were then performed. Full details of these results are provided in Appendix C. Summary information however on the sequencing and alignments is shown in table 3.

IMAGE clone 3473035 was found by sequencing downstream (primer RapBsF, then RapBsF2) and upstream (primer RapBsR) to contain an incomplete protein coding sequence starting downstream of the translation start site. Consequently a further EST was identified from the databanks,

BG731378 which overlapped the 5' region of the sequence generated from IMAGE clone 3473035 (*i.e.* EST BI444610) and which was used to identify a third IMAGE clone to complete the sequencing upstream of that in IMAGE clone 3473035. This overlap is also shown in Appendix B.

Table 3 : Sequenced p52 candidate clones — summary information						
				Protein sequence homology ³		
IMAGE Clone no.	EST accession no(s) ⁴ used for sequencing	Transcribed length (nucleotides)	M _r (KDa)	XRAPA/B	RAP55 (Q9YH12)	Q9BX40 ⁵
3405473 (XRAPA)	BG408666, BG486665	1733	51.3KDa	58% similarity 48% identity	82% similarity ⁶ 74% identity	54% similarity 46% identity
3473035 (XRAPB)	BI444610, BG731378	2187	45.7KDa		55% similarity 46% identity	75% similarity 71% identity
<u>Additional homology information</u>						
XRAPA	Translation identical to <i>Xenopus</i> protein Q8AVJ2 (UNIPROT), 51.3KDa protein expressed in the embryo. IMAGE clone no. 5571036.					
XRAPB	Homology to Q9BX40 (UNIPROT), human protein, 42KDa.					
<u>EST information</u>						
BG408666	Expressed from <i>Xenopus</i> oocytes. Sequence generated from IMAGE clone 3405473. Similar to 5' of cDNA for Q9YH12.					
BG486665	Expressed from <i>Xenopus</i> oocytes. Sequence generated from IMAGE clone 3405473. Similar to 3' of cDNA for Q9YH12.					
BI444610	Expressed from <i>Xenopus</i> oocytes. Sequence generated from IMAGE clone 3473035. Similar to 5' of cDNA for Q9YH12.					
BG731378	Expressed from <i>Xenopus</i> lung tissue. Sequence generated from IMAGE clone 4674033. Similar to 5' of cDNA for Q9YH12.					

³ Using EMBOSS-Align tool at EBI website

⁴ EBI: www.ebi.ac.uk. All ESTs are *Xenopus* cDNA clones with similarity to Q9YH12.

⁵ UNIPROT accession number for protein with 53% similarity and 45% identity to RAP55

⁶ Similarity refers to percentage of nucleotides over the alignment that have an EMBOSS-align algorithm 'score' greater than 0 (see Methods and Materials).

XRAPA contains a coding sequence for a 471 amino acid length protein compared to XRAPB which encodes a protein of 420 amino acids. This corresponds with proteins of predicted molecular weight of 51.3KDa and 45.7KDa respectively. Based solely on the predicted mass therefore, the band just below the 53KDa marker in figure 35 potentially represents XRAPA expressed from the pCMVSPORT6 vector which is recognised by the anti-RNP2(p52) antibody. The predicted molecular mass of XRAPB does not correspond to the apparent size of any reported protein bound to by anti-RAP55 or anti-RNP2 antibodies.

The transcribed sequence of XRAPA is revealed as being nearly identical (3 nucleotides different) to that of another cloned *Xenopus* cDNA (IMAGE: 5571036) that is expressed in the embryo and also encodes a protein of identical sequence to that expressed from IMAGE clone 5571036, Q8AVJ2.

The translated sequence of XRAPB but not XRAPA shows identity to the peptide sequences obtained from p52 extracted from SDS-PAGE gels running oocyte poly(A)+ material (J.Sommerville, unpublished). This suggested that the alignment performed on the translated sequences of XRAPB and the human RAP55-like protein Q9BX40 (that in the unpublished data also showed this similarity to p52) was warranted. As

expected XRAPB has a high peptide identity and similarity to Q9BX40 (table 3, Appendix C) with respective values of 71% and 75%. However, an alignment of XRAPB with the protein sequence of RAP55 reveals a relatively much lower similarity and identity. As expected, similarly low values were obtained for an alignment between Q9BX40 and RAP55 (table 3 footnote, Appendix D).

Thus although XRAPA may be closer in predicted molecular mass to the apparent mass of p52, XRAPB appears far more likely to represent this particular component of *Xenopus* mRNP particles yet seems likely to not represent a *Xenopus* orthologue of RAP55. Accordingly the alignment between XRAPA and Q9BX40 shows relatively low similarity and identity. Further analysis on the amino acid composition and charge distribution of XRAPB is presented in Appendix B.

The alignment of the protein sequences of XRAPA and RAP55 however, in contrast to XRAPB, have a high similarity and identity with values of 82% and 74% respectively (table 3, Appendix C). Also the predicted molecular mass of XRAPA (51.3KDa) is very close to that of RAP55 (51.9KDa) although the apparent mass in electrophoresis of RAP55 is 68KDa. Thus, XRAPA is theoretically far more likely to represent an orthologue of RAP55 even though the apparent electrophoretic mass of XRAPA is not as

yet determined.

These alignments therefore suggest that a *Xenopus* RAP55 orthologue and p52 are separate proteins. In line with this apparent distinctiveness a low similarity and identity was found on aligning the protein sequences of XRAPA and XRAPB (58% and 48% respectively; table3, Appendix C).

Given the much higher homology of XRAPA with RAP55 it was thought useful to examine the protein sequence for features identified by Lieb *et al.* (1998) in RAP55.

At the amino terminus of RAP55 are two proline-rich domains surrounding a serine/threonine-rich region. Towards the C-terminus are two arginine/glycine-rich regions (RGG boxes). The functions of these domains in RAP55 remain theoretical based on consensus with their functioning in other proteins. However, structural similarities between XRAPA and RAP55 strengthen the case for XRAPA as a RAP55 orthologue in *Xenopus*. The corresponding regions in XRAPA to those identified by Lieb *et al.* (1998) in RAP55 as determined by the alignment in Appendix C are highlighted in fig.36.

MSGGTPYIGS	KISLISKAEI	RYEGILYDID	TENSTVALAK	VRSFGTEDRP	TDRPIPPRDE	60
<u>VFEYIIFRGS</u>	<u>DIKDLTVCEP</u>	<u>PKPQCCLPQD</u>	<u>PAIVQSSLGS</u>	<u>SSASSFQSVS</u>	<u>SYGPFGRMPT</u>	120
<u>YSQFSTSPLV</u>	<u>GQQFGAVAGS</u>	<u>SLTSFGAETT</u>	<u>SSTSLPPSSV</u>	<u>VGSTFTQEAR</u>	<u>TLKTQLSQGR</u>	180
<u>SSSPLDLRLK</u>	<u>SPTIEQAVQT</u>	ASAPHPSSA	AVGRRSPVLS	RPLPSSSQKT	AESPEQRKGE	240
LHKIQRPDTE	QKNDYKNDLS	RRQPVLAAQ	<u>PRRGRGGNRG</u>	<u>GRGRFGVRRD</u>	<u>GPMKFEKDFD</u>	300
FESANAQFNK	EDIDREFHNK	LKLKDDKPEK	PLNGEDKTDS	GVDTONSEGH	AEEDVLAAG	360
VCYDKTTSF	FDSISCDNR	DRRQWAEER	RMNAETFGLP	LRSNRGRGGY	<u>RGRGGMGFR</u>	420
<u>GGRGRGGERR</u>	<u>GAPGGVGGFG</u>	<u>PSRGRGGSR</u>	<u>GGRGREFAE</u>	YEYRKDNKVA	A	471

Figure 36: Consensus regions of XRAPA protein sequence.

Underlined amino acids indicate proline-rich domains; amino acids highlighted in green indicate a serine/threonine-rich domain and red-highlighted areas indicate RGG boxes. All domains shown were identified from the alignment (in appendix C) with corresponding regions identified in RAP55 by Lieb *et al.* (1998).

As shown in fig.36, the alignment of XRAPA and RAP55 (Appendix C) enables identification of equivalent domains, originally identified by Lieb *et al.* (1998) in RAP55. The first proline-rich (P-rich) region of XRAPA is identical to that of RAP55 as is the first RGG box. The second proline-rich region is the domain that most diverges from the equivalent in RAP55 with a lower proline content but has several stretches of identity nevertheless. The second RGG box in XRAPA is also divergent with a higher glycine (G) content but identical arginine (R) content. The serine/threonine-rich region of XRAPA is also relatively divergent in sequence but over the domain has virtually identical serine (S) and threonine (T) content. Thus XRAPA, in similarity to RAP55, appears to have a modular structure with

distinct consensus domains.

Although there is a similar proline-rich region in XRAPB compared to RAP55 near the N-terminus, the serine/threonine-aligned region contains far more mismatches than in the XRAPA/RAP55 alignment. Also the second proline-rich domain and RGG boxes of RAP55 when aligned to XRAPB contain several large gaps. Therefore in view of the relatively much lower similarity and identity of XRAPB and RAP55 compared to XRAPA, XRAPB appears to have diverged from *Pleurodeles* RAP55 much further than XRAPA.

Discussion

Storage of mRNA as mRNP is common amongst vertebrates during germ cell formation for use during early development. In *Xenopus* for example mRNP is accumulated in large quantities to provide mRNA for translation when the rapid cell divisions and associated repeated condensations and expansions of chromatin of early embryogenesis in effect suspend transcription. High rates of transcription up to the third stage of oogenesis in *Xenopus* accumulate the large mRNP pool after which point transcription rates decline. The mRNP can then be used to encode protein syntheses during oocyte maturation, fertilisation and embryogenesis.

The composition of *Xenopus* mRNP particles has been well studied (Darnborough and Ford, 1981; Ladomery *et al.*, 1997; Lieb *et al.*, 1998) and the focus of this study has been on one of the four most abundant mRNP components, RNA helicase Xp54, an ATP-dependent helicase of the DEAD-box family with orthologues throughout metazoans and in yeast.

Specifically, this study has examined the effect of Xp54 on the nuclear export and stability of maternally transcribed mRNAs and the effect of FRGY2 — another abundant mRNP protein — with which Xp54 is

understood to interact and work cooperatively in the formation and functioning of mRNP for the purposes of translation control (Sommerville, 1999).

An apparently differential nature of association and therefore influence of Xp54 is revealed amongst certain mRNAs along with a more general influence on mRNA stability in the oocyte. The apparent influence of the mRNP masking proteins FRGY2a and FRGY2b on oocyte mRNA stability revealed by this study is also discussed.

The steady-state levels of mRNAs during oogenesis

In order to form a basis for investigating the influence of Xp54 on specific mRNA stabilities it was deemed necessary to examine the changes in levels of specific mRNAs during oogenesis both to reveal any differences that might exist in their presence or absence and differences in the quantities present during the course of oogenesis.

Both the mRNA quantities present per mass of oocyte and per single oocyte during oogenesis were investigated but in most cases — encompassing both non-translating and translating mRNAs — a consistent trend of increase per oocyte over the five stages was seen, although with variable degrees of increases between the individual stages. It should be noted though that certain different mRNAs seem to exist in clearly

different total quantities at particular stages in oogenesis. For example, *FRGY1* shows a much higher presence at stage 3 of oogenesis than do *rpL1* and *mos* and *cyclin B1* shows a particularly highly level of mRNA at stage 6 of oogenesis.

Overall however, it seems more logical that there should be a gradual increase in most mRNAs rather than a possibly more wasteful fluctuating increase then partial loss. Whereas non-translating genes would be expected to be solely accumulating mRNA as mRNP for later translation, the mRNA for translating genes would be thought to also partially comprise mRNA that is about to undergo translation to provide for an ongoing need for its protein in cellular functioning. Furthermore although the mRNP pool is understood to be mostly formed by stage 3 of oogenesis (Sommerville, 1981), the results in figure 14 suggest that transcription and an accumulation of diverse mRNAs continues towards the end of oogenesis.

Despite the long-term accumulation of mRNAs from a wide range of genes, figure 15 suggests that mRNA stability varies according to the particular gene rather than according to its translational status. *FRGY1*, *mos*, *histone H4* and *rpL1* represent mRNAs from distinctly different classes of gene in that *FRGY1* protein is not detectable until after oocyte fertilisation, *Mos* is not detectable until oocyte maturation whereas *rpL1*

and histone H4 are translated from early oogenesis as a ribosome and chromatin component respectively (J.Sommerville, pers. comm.).

However, the genes of all these proteins are apparently dependent on transcription at stage 4 of oogenesis for their continued presence and in the absence of transcription — whether through direct inhibition of RNA polymerase II action on these genes or indirect means — their mRNA levels decline until they are undetectable. *Histone B4* mRNA however, although apparently affected in its net stability by transcription inhibition, remains detectable during the course of the experiments as seen in fig.15 suggesting that *histone B4* mRNA is stabilised to a greater extent, possibly by mRNP storage or that, in contrast to the other genes analysed in fig.15 at this stage of oogenesis, there is mRNP storage of *histone B4* mRNA. For most genes analysed in fig.14, large increases in mRNA accumulation begin only between stage 4 and 5 of oogenesis which, taken with the instability of mRNA from genes such as *rpL1* and *histone H4*, might suggest the possibility of storage of these genes as mRNP beginning later than is currently understood to be the case. Storage for *mos* and *histone B4* mRNA would be expected given the lack of detectable Mos and histone B4 protein before oocyte maturation (Sagata, 1997; Hock *et al.*, 1993). The same might be thought to apply to *FRGY1* mRNA as well but is only implicated for *histone B4* mRNA. This raises the question of what is

happening to mRNAs such as *mos* and *FRGYI* which are not understood to be translated but are transcribed apparently without such storage, at least according to fig.15. One possibility is some processing event occurs to such mRNAs which renders them un-translatable and not subject to mRNP storage. The published nucleotide sequences for many non-translating genes indicate a lack of introns suggesting splicing could contribute the cell's discrimination between these mRNAs and mRNAs destined for translation. However, the apparent shared instability between non-translated and translated mRNAs and the presence of introns in the *histone B4* sequence weighs against this idea. Alternatively, sequence signals within the message — perhaps the untranslated regions — might enable binding of inhibitory proteins or binding of proteins in a way so as to inhibit translation but not mask and stabilise the message. Thus such non-translating messages may transcribe and share aspects of stability as translating messages as indicated respectively by fig's 14 and 15 but be rendered relatively inert. In any case these are processes which must presumably be overcome or avoided after maturation or fertilisation. In summary, the set of analyses presented in fig.15 could be said to indicate differences in stability between mRNAs irrespective of whether or not they are understood to become mobilised only subsequent to the induction of maturation.

It is interesting to note that two of the three mRNAs for those genes whose translation begins after maturation initiation in figure 16 are those which show least evidence for becoming associated with Xp54-containing structures *i.e.* *mos* and *cyclin B1* mRNA. The situation could be that most of the total quantity of these mRNAs were already stored in Xp54-containing mRNP particles. However the similarities in stabilities between *rpL1* and *mos* mRNA in fig.15 — both disappearing when transcription is inhibited — suggests that association with Xp54 at this stage in oogenesis is not for the purpose of long term storage as mRNP.

Xp54's influence on the nuclear export of mRNA

Previous published work on the intracellular movement of Xp54 indicates it is a shuttling protein which is imported into the nucleus following synthesis where it binds to transcripts and is then exported as mRNP by a mechanism that is not fully understood, or is exported if in excess of the available mRNA molecules (Smillie and Sommerville, 2002). The significant quantity of T7-tagged wild-type Xp54 in stage 3 nuclei previously reported by Smillie and Sommerville is borne out in figure 17. However the introduction of a mutation into the DEAD box motif of the core helicase region of Xp54 apparently interferes with the shuttling of the protein. Although the translation of the DQAD T7-Xp54 variant seems to

be less than the wild-type at both time-points, the accumulation of the protein in the nucleus appears to be particularly affected for the DQAD form. Given that stage 3 oocytes are transcriptionally active and Xp54's import and shuttling function is understood to be maximal during this early oogenesis period, it appears that the DEAD motif contributes to the import of the protein into the nucleus, though enhancing the efficiency of import rather than being crucial for it.

The DEAD-box motif is thought to be essential as part of an ATPase domain in DEAD-box containing proteins based on its established function in the translation initiation factor eIF4A (de la Cruz *et al.*, 1999). The DEAD consensus sequence works with other motifs including the recently identified Q motif (Tanner *et al.*, 2003) in this domain in effecting ATP binding and hydrolysis and may bring about conformational change in the protein. Thus whether or not there is conformational change of Xp54 in its association with any import factors during nuclear import as there is thought to be during helicase functioning it is likely that the DEAD-motif is operating as part of a larger region within Xp54's structure and an amino-acid substitution may weaken this operation in some way.

The first usage of RT-PCR to gain an insight into the relative quantities of specific mRNAs in the oocyte (fig's 20 and 21) gave a set of individually distinctive results but certain effects were apparent and shared between

certain genes as a consequence of exogenous Xp54 overexpression. Indeed, the expression of Xp54 from microinjected expression vectors seems to be an effective way of achieving overexpression of Xp54 (fig.18) with the exogenous form apparently complementing endogenous Xp54 expression rather than replacing it by, for example, competition for expression factors such as translation machinery. The apparent effects on the export of individual mRNAs as a consequence of expression of exogenous Xp54 forms are summarised in table 4.

A quite clear enhancement of nuclear export is seen in the ratio of cytoplasmic:nuclear mRNA for the two ribosomal protein genes analysed — *rpL1* and *rpS1* — and for *cyclin B1* when wild-type Xp54 is overexpressed (fig.20a). For these same genes, the expression of the DQAD form of Xp54 seems to have a less marked effect on this mRNA nuclear-clearing effect than overexpression of the wild-type form although occasionally seems to enhance export relative to the uninjected oocyte sample. However, for *rpS1* and also *FRGY1*, expression of the DQAD form may be inhibiting the export of the mRNA from the nucleus. The protein evidence for the effect of the DQAD mutation on Xp54 intracellular movement (fig.17) suggests a reduction in its import efficiency at this stage of oogenesis. Therefore this disruption to the shuttling of Xp54 caused by the mutation is perhaps borne out to varying

degrees in the intracellular distribution of the specific mRNAs stated in table 4. It is not yet known if Xp54 is essential for the export of mRNA to the cytoplasm as the nuclear export motif that enables export of the protein alone does not seem to operate when Xp54 is mRNA-bound (Smillie and Sommerville, 2002). Therefore it is unclear whether the DQAD mutation to the DEAD-box motif is inhibiting export of Xp54 bound to mRNA-containing particles or whether the reduction in export of certain mRNAs is related to the impaired shuttling ability of Xp54 caused by reduced import as suggested by fig.17. The location of the DEAD-box in an ATP binding and hydrolysing region of the helicase however suggests that any reduction in mRNA export caused by the mutation may more likely be due to a defect in the ATP-requiring helicase activity that it might demonstrate during the packaging of mRNA in the nucleus when proteins such as the masking proteins FRGY2a/b associate to help form the mRNP particle (Ladomery *et al.*, 1997). Thus less mRNA would be exported through the mRNP route in DQAD-*Xp54* injected oocytes relative to wild-type *Xp54*-injected oocytes as a result of the mutated protein competing with endogenous wild-type *Xp54* to bind with mRNA molecules although this inhibitory effect would be expected to be more pronounced in stage 2 oocytes, for example, when transcriptional activity is higher. Any enhancement in specific mRNA export brought about by expression of

exogenous DQAD-Xp54 relative to the uninjected samples is thought to be a result of a greater availability of Xp54 to associate and package these mRNAs for export albeit with impaired function compared to the oocytes injected with wild-type *Xp54* DNA.

In terms of the current understanding of bulk mRNA export, it is not yet fully understood how an mRNP particle containing such abundant proteins as Xp54 and FRGY2a/b and any other masking proteins and regulators fit into the scheme of binding of the postulated essential, central export factors sub2p/UAP56 and mex67p/TAP. Xp54 can be detected around chromatin and is found in the same cellular locations and extracted fractions as the Y-box masking proteins FRGY2a/b which Xp54 assists to bind to structured RNA molecules *in vitro* (Ladomery *et al.*, 1997; Smillie and Sommerville, 2002; Sommerville and Ladomery, 1996). This suggests that these abundant mRNP proteins bind at around the same time of any export determinants that bind to mRNA during transcription or at around the same time of factors that determine whether the mRNA needs to be spliced before being allowed to proceed with the export process and bind prior to the binding of export determinants that might be associated with 3' end formation and the association with mex67p/TAP (reviewed in Cullen, 2003).

Therefore it is envisaged that the overexpression of Xp54 in those cases

where it enhanced the export of a specific mRNA is most likely to be a consequence of the greater availability of Xp54 to assist in the packaging of mRNA and hastening of the export process rather than disrupting or otherwise targeting another stage of the process. In a biological system so heavily dependent on the storage and repression of mRNA for later use it seems highly plausible that Xp54 plays a vital role in the assembly of mRNP particles for nuclear export and storage in the cytoplasm, even in stage 4 oocytes when transcription is understood to be gradually decreasing (although the continued accumulation for the genes of different translational status in fig.14 would suggest that transcription is actively contributing to the stored pool of mRNP at this stage). In order to enhance export of selected mRNAs Xp54's importance must be such that a lack of the protein would be expected to inhibit the export of the same mRNAs through some indirect contribution to the export process. This contribution is thought to be the necessity for Xp54 for packaging a fraction or all of the transcripts of a particular gene as mRNP before nuclear export. An effective reduction in the availability of fully functional Xp54 for essential packaging through competition with the DQAD-mutant form may be the cause of those cases where suspected inhibition of export by the mutant is more noticeable than an enhancement in export by the wild-type as seen in *rpS1* and *FRGY1* in this study. In those cases — *histone B4* and *histone H4*

— where the expression of either form of Xp54 in the oocyte did not clearly indicate Xp54-enhanced export or DQAD-mutant inhibited export, it is concluded that the availability of Xp54 is not as critical to the efficiency of export in the form of mRNP. This could be due to lower normal rates of transcription at the corresponding chromosomal loci for these genes although this is difficult to prove as neither the mRNAs which appeared to be enhanced in their export by overexpressed Xp54 or those that didn't have begun their sharp increase in mRNA per oocyte at stage 4 (fig.14, right-hand graphs). Also figure 15 indicates that *histone H4* may indeed have a higher transcription rate than *L1* as judged by the quicker recovery levels of its mRNA after apparent abolishment of the presence of the respective mRNA levels of both genes. As described below, *histone H4* mRNA levels do not respond to lowered Xp54 levels after maturation induction in the same way as do other genes that have been translating before maturation but closer to the way *histone B4* and other previously non-translating genes behave (fig.28) suggesting the dependency on Xp54 for efficient nuclear export can vary in spite of translational status of the mRNA.

In conclusion then, it seems that Xp54 influences in distinct ways the rate of export of mRNAs encoded by genes required for a range of functions including ribosome structure formation (*rpL1*) and, in the case of *FRGY1*,

somatic cell translation regulation or transcription regulation of Y-box containing genes in the oocyte (Sommerville and Ladomery, 1996). It is interesting to note that these genes include those mRNAs that are translated both before (*rpL1*) and after (*FRGY1*) oocyte maturation thus underlining the wide range of targets that Xp54 apparently has. Also this evidence could be construed as supporting the idea that mRNAs are not packaged into mRNP particles indiscriminately but selectively: either alone or with mRNAs from genes whose export and subsequent regulation can be shared to a certain degree.

<u>Presumed effect of overexpression of Xp54</u>	<u>Gene mRNA produced from</u>
Export enhancement	<i>rpL1, rpS1, cyclin B1</i>
Export reduction by DQAD mutant	<i>FRGY1, rpS1</i>
Stability enhancement	<i>cyclin B1</i>
Stability reduction	<i>rpL1, histone H4</i>
Stability reduction by DQAD mutant	<i>FRGY1</i>
Table 4: Influence of Xp54 (or the mutated DQAD form where stated) on specific mRNAs analysed in this study.	

Xp54 and stability of mRNA

Overexpression of Xp54

In addition to the role of Xp54 in the formation of mRNP particles for storage after export to the cytoplasm, its continued association with the particles as opposed to a possibly more efficient, in cellular energy terms, recycling of the protein back to the nucleus suggests a function for Xp54 in the cytoplasm. The high degree of amino-acid sequence similarity of Xp54 with the yeast translation initiation factor eIF4A when sequence-aligned (Ladomery *et al.*, 1997) supports the idea that this cytoplasmic role may be in removing structured areas in the 5' UTR of mRNA (or further downstream) to facilitate the passage of the ribosome when the mRNAs are mobilised from the mRNP by the oocyte for translation.

Thus, in view of the more likely nuclear role of Xp54 being in facilitating mRNP assembly for export rather than mediating export itself, then when examining the combined nuclear and cytoplasmic densitometry values for the same genes described above any differences between samples in terms of the net stability of mRNA would be likely to give an indication of Xp54's combined influence on transcription and export and influence within cytoplasmic mRNP particles constituting most of the mRNA that is translationally repressed at this stage of oogenesis.

This difference on net stability is most striking for *rpL1* and *cyclin B1* mRNAs and the main effects of Xp54/DQAD-mutated Xp54 in terms of stability are summarised in table 4. For *rpL1*, overexpression of Xp54 results in a destabilisation of the mRNA whereas a stabilisation is found in *cyclin B1* mRNA, both relative to the DQAD-mutant samples and the uninjected samples. In addition however, the mutant form has the opposite effect for each gene relative to the uninjected (except the 48 hour *cyclin B1* sample) and the wild-type overexpression samples thus providing strongest evidence from the genes analysed for the differential role of Xp54 according to the gene. That is, a stabilisation of *rpL1* mRNA by DQAD-Xp54 expression and destabilisation of *cyclin B1* mRNA.

Therefore, in spite of the export enhancement seen for both genes in the presence of ectopic Xp54 expression, notably contrasting effects are seen on the total stability. In other words, even in the presence of a presumably functionally impaired Xp54 enzyme competing with endogenous wild-type Xp54 the export process is inhibited for both mRNAs whereas the overall quantities of the particular mRNAs at the time of sampling differ. The main difference between the *rpL1* and *cyclin B1* results is that the effect of export inhibition by the DQAD-Xp54 for *rpL1* seems to be that in spite of the movement of less mRNA to the cytoplasm there is a notably greater stability of the mRNA that remains in the nucleus relative to the wild-type

injected samples. For *cyclin B1* mRNA however, there is no such nuclear stabilisation by the DQAD-Xp54 and the cytoplasmic mRNA quantity remains lower than in the wild-type injected samples. Thus there does not appear to be a simple relationship between Xp54's role in export and the eventual net stability of an mRNA in the oocyte.

One possible explanation for this may be the different translational status of the two genes in question is that *rpL1* mRNA is translated before maturation but *cyclin B1*, a gene required for meiotic progression during maturation, is not translated until maturation's onset. Whilst in stage 3 oocytes when the stored maternal mRNP pool is understood to be mostly established one would expect a declining rate of accumulation of *cyclin B1* mRNA for translation at the onset of maturation, it does not have the far wider, structural role that *rpL1* has to continually provide functioning ribosomes for the greater dependency on translation that arises at maturation and so a much greater accumulation of *rpL1* mRNA might be expected for storage and immediate translation. Taking this to be the case and in view of the apparently differential nature of Xp54's role in mRNA export one can speculate that the assembly process of which Xp54 is an integral part simply might favour the packaging of certain more abundant mRNAs at the expense of the less abundant ones which consequently show less 'blockage' in the nucleus. However the pattern of accumulation of *rpL1*

and *cyclin B1* indicated by fig.14 does not corroborate this so it is possible that Xp54 may bind to both non-translating and translating mRNAs and that certain genes, perhaps translating ones, are somehow more dependent on fully functional Xp54 for efficient nuclear export.

This would lead to accumulation of nuclear mRNA over the sampling period whilst nuclear export was impaired by the presence of the DQAD-Xp54. Thus it is concluded that for a translated gene whose efficient export depends on Xp54, a greater total stability is detected in DQAD-Xp54 injected samples owing to the reduced efficiency of transport and the greater accumulation in the nucleus and therefore less passage to the cytoplasm for storage in turn meaning a lower amount of non-masked mRNA is being exported and thus not utilised. For a gene such as *cyclin B1* there is less nuclear accumulation of mRNA in DQAD-Xp54 injected samples and, presumably, no utilisation and subsequent degradation of mRNA in the cytoplasm occurs in wild-type Xp54-injected samples thus leading to the lower total stability in DQAD-injected samples relative to wild-type Xp54-injected samples.

The rpL1 pattern is seen, albeit less convincingly, for rpS1 mRNA (fig.20). *Ribosomal protein S1*, being a gene translated before maturation, shows greater stability in DQAD-Xp54 sample relative to wild-type Xp54 samples and the apparent inhibition in nuclear export is observed as a high

nuclear mRNA quantity. Where there is not such a striking indication of exogenous Xp54-enhanced nuclear export as there is for mRNAs such as *cyclin B1* and *rpL1* this bottleneck nuclear accumulation of mRNA hindered in its export by DQAD-Xp54 relative to the wild-type injected samples is not seen. Thus, *FRGY1* mRNA is not translated until after fertilisation and although it does not show such a clear enhancement of export by ectopic Xp54 expression and blockage by DQAD-Xp54 as does *rpL1*, nevertheless shows a lower total stability in the DQAD-Xp54 samples relative to the wild-type *Xp54*-injected samples (fig.21). This, in view of the conclusion outlined above, is presumably due the combined effects of a lower or nil usage of the mRNA following nuclear export in the wild-type *Xp54*-injected samples and less dependency on Xp54 for efficient nuclear export. Thus there is more nuclear mRNA in the wild-type *Xp54*-injected samples and less nuclear accumulation of mRNA in the DQAD-*Xp54* injected samples compared to *rpL1*. Figure 15 indicates however that *FRGY1* mRNA shares the stability of *rpL1* mRNA in that without transcription after 12 hours it all degrades suggesting that a lower stability in DQAD-*Xp54* injected samples relative to wild-type *Xp54* injected samples is more related to a reduced dependency on Xp54 for efficient nuclear export with less nuclear accumulation in DQAD-injected samples accordingly relative to DQAD samples of a gene such as *rpL1*.

The effect on general transcription rates or transcription rates at specific loci, if any, that would be exerted by ectopic Xp54 expression or the presence of a mutated Xp54 in the nucleus is not known. As stated, Xp54 associates with lampbrush chromosomes to bind to nascent transcripts in transcriptionally active oocytes and this association can be stimulated by activating transcription in stage 6 oocytes normally quiescent in terms of transcription (Smillie and Sommerville, 2002). The re-association with nascent transcripts is thought to be partly due to the release of Xp54 from a cytoplasmic anchoring mechanism that is dependent on the carboxy-terminus of Xp54 but there is not thought to be any influence on chromatin structure from Xp54 that would facilitate dissociation of emerging mRNA although the sheer amount of Xp54 present in the vicinity could conceivably impair the progress of factors involved in transcribing a gene. Similarly although Xp54 is thought to be involved in the efficient export of mRNA in the transcriptionally active stages of oogenesis, it is not thought likely that this involvement would extend to some of the processes outlined in Introduction that contribute to mRNA export such as 3' end formation where aberrant polyadenylation in yeast can lead to the mRNA being retained in the nucleus for degradation by complexes of exonucleases in the nucleus known as nuclear exosomes (Hilleren *et al.*, 2001). In other words, the effect of Xp54 on transcription and export is

thought more likely to be less direct and specific.

Underexpression of Xp54

Having seen that overexpressing Xp54 or DEAD-box mutated Xp54 in the oocyte affects the stability and distribution of specific mRNAs and that Xp54 with a wild-type DEAD-box motif seems essential for the efficient export of some of them and for efficient import of the protein itself, it is reasonable to assume that a reduction in cellular Xp54 would also affect the intracellular transport and stability of oocyte mRNAs.

An effective reduction in exogenous T7-tagged Xp54 expressed in the oocyte is brought about by the antisense morpholino that is targeted against the translation start region of Xp54 mRNA when cytoplasmically injected and can be maintained for at least 36 hours (fig.23a and fig23b).

The greater apparent effectiveness of the morpholino in the cytoplasm probably relates to the poor diffusion of antisense oligonucleotides including morpholino variants across cell membranes (Akhtar and Wickstrom, 1991; GeneTools technical data sheets). Thus it is likely that the *Xp54* antisense morpholino binds to the T7-Xp54 message when it emerges in the cytoplasm non-masked which is assumed to occur along with any possible storage of masked *Xp54* mRNA given the presumed continual need for the protein in assembling masked mRNP particles

throughout oogenesis.

The anti-*Xp54* antisense morpholino is also apparently able to impact upon the oocyte's pool of endogenous *Xp54* (fig.23d) as judged by the decline in total *Xp54* signal 12 hours after antisense injection compared to the gradual accumulation in *Xp54* following injection of the presumed inert control morpholino. In light of the apparent phenomenon whereby the injection process provokes a response that somehow leads to increased translation of *Xp54* as is apparently occurring in the control immunoblot of fig.23(d) compared to the relatively steady-state of *Xp54* in non-injected oocytes (fig.23e), it is likely that it is the sequence of the anti-*Xp54* antisense morpholino that is responsible for a decrease in endogenous *Xp54* immunoblotting signal. The inhibition of translation by the antisense morpholino apparently takes over 6 hours to make its impact evident but the *Xp54* level after this time remains lower than the 6 hour time interval for at least 48 hours post-injection. Given the apparent limited stability of *Xp54* as seen with cycloheximide-treated oocytes in fig.23(e) whereby the impact of *Xp54* degradation seems to be visible within the time-frame of the experiment, it seems likely that natural *Xp54* decay contributes to an extent to the relative reduction in endogenous *Xp54* levels seen in antisense morpholino-injected oocytes (fig.23d) as translation of fresh *Xp54* is concurrently inhibited. This would be thought to be behind the

prevention of accumulation of Xp54 in antisense morpholino-injected oocytes in contrast to the control immunoblot or the relatively more steady-state seen in non-injected oocytes (fig23e).

A low nuclear presence of *Xp54* mRNA detected by RT-PCR (fig.23c) is not surprising given the low overall presence of the whole transcript in total mRNA detected previously in stage 4 oocytes (Ladomery *et al.*, 1997). However, the apparent stabilisation of the *Xp54* transcript in morpholino-injected oocytes can be interpreted as inhibition of translation and the associated subsequent decay of mRNA which remains available as a template for amplification by RT-PCR after extraction. Alternatively, as stated in Results, the reduction in cellular Xp54 brought about by the morpholino could somehow be responsible for the stabilisation of the transcript thereby producing more copies of Xp54 protein itself.

The morpholino sequence includes a part of the T7-sequence that is tagged to the cloned *Xp54* DNA sequence on the plasmid vector so the effecting of a change in endogenous oocyte *Xp54* mRNA levels together with the significant and lasting reductions in exogenous T7-tagged Xp54 expression presented in figure 23 are taken as further confirmation that the morpholino is altering, most likely reducing, endogenous Xp54 levels through blockage of *Xp54* mRNA translation. Thus it is thought that the postponement or prevention of normal decay of *Xp54* unmasked transcript

in the cytoplasm by the morpholino is at least partly a cause of the increase in stability seen in the AS1C and AS2C samples of fig.23(c).

Given that Xp54 is continually present into early embryogenesis stages and a fraction of the total cellular amount is present in the nucleus in an RNA-free form involved in mRNP assembly — at least in early oogenesis (Ladomery *et al.*, 1997) — it would be thought that in stage 4 oocytes there would be a degree of Xp54 mRNA translation occurring to maintain Xp54 levels through oogenesis and possibly some formation of Xp54 mRNA-containing mRNP particles although the mRNP pool is, for the most part, known to be established before stage 4. The question is whether a morpholino-induced reduction in cellular Xp54 is stabilising cellular Xp54 mRNA in addition to the direct effect of the morpholino and whether or not part of the Xp54 mRNA translation is from masked mRNA. If a morpholino-induced reduction in translation of Xp54 from non-masked Xp54 mRNA occurs then under a scenario where masked mRNA also is being mobilised onto polysomes (*i.e.* unmasked) there would be less masked maternal mRNP particles formed containing Xp54 mRNA available and so the reduction would be perpetuated. That is, less Xp54 to form Xp54 mRNA-containing mRNP and so less mRNP to contribute to its replenishment so even if unmasking was occurring, rather than stabilising its own mRNA, Xp54 would be reducing the amount stored. Release of

translational repression maintained by masking proteins is thought to require their dephosphorylation in response to progesterone stimulation of the oocyte (Sommerville and Ladomery, 1996) and not by selective internal mechanisms during oogenesis. Thus mRNAs for essential translation to maintain the cell during oogenesis are understood to be exported from the nucleus without FRGY2a/b binding in such a way as to mask to the mRNA, in a poorly understood process. In view of all of this, it seems less likely that the stabilisation of *Xp54* mRNA is indirectly brought about by the anti-*Xp54* morpholino in reducing the amount of *Xp54* available and that the *Xp54* mRNA-containing mRNP particles — mostly all formed by stage 4 of oogenesis — are probably like any other mRNP particle in being repressed until maturation/early embryogenesis. Having confirmed the efficacy of the anti-*Xp54* morpholino's action on the target mRNA sequence, it seemed likely that a differential range of effects would be observed on cellular mRNAs as a result of reduced *Xp54* presence as were seen as a result of its enhanced presence.

The densitometry of the RT-PCR replicates shown in fig.24 give an indication of the repeatability of the RT-PCR process and subsequent analysis. In general the patterns of change across the 48 hour analysis period are consistent, with a rise or fall in mRNA presence between the time points being matched between the three runs of the RT-PCR

reactions. However, it is clear that significant amount of stringency must be practised in the loading of RNA samples and other reaction components into RT-PCR reaction tubes and in the loading of the cDNA into the agarose gel as errors at these stages are thought to be responsible for changes in densitometry value between samples for consecutive time points which do not follow the trend of the other two sets of reactions (notably early time points in the first runs of *mos* and *FRGY1* RT-PCRs). All densitometry values for each gene analysis presented in fig.24 were normalized to the first control (C0) which was given a value of 1 thus allowing all three sets of values obtained for a gene analysis to be on the same scale. In this way differences in the brightness, contrast and/or lighting conditions for the photography process and consequently affecting the densitometry gel-image scanning process between the three sets of RT-PCR reactions could be, in effect, nullified. It also allows the scale of change across the time points to be compared with more accuracy between the separate gene analyses.

In doing this normalization however, underloading errors in the C0 sample for one of the runs would give a set of normalized values that were uncharacteristically higher than the C0 compared to the other two runs which is thought to have probably been the case for one set of values in the *rpL1* and *FRGY1* analyses. However, as stated in Results this would not

affect the pattern of change between the samples for the remaining time points. Normalization was similarly applied to the results presented in fig.25 for the same reasons as it was for those in fig.24 although any normalization inaccuracy would not be apparent as the RT-PCR reactions were performed once for each gene.

It is thought most likely that comparing the data obtained for the different genes from the morpholino treated oocytes at 48 hours is most informative as control samples exist for all genes analysed at this time point. Though the samples for the intermediate time-points in fig.24 (6, 12, 24 and 36 hours) may or may not reflect the indirect influence of the anti-*Xp54* morpholino they are worthy of note as they illustrate the diversity in the fluctuating patterns of mRNA levels in the five genes presented.

Thus the mean changes shown may be partly or mostly a result of a morpholino-induced reduction in *Xp54* presence or of little consequence of a reduction in cellular *Xp54*. The examination above of the *Xp54* overexpression and DQAD-*Xp54* expression results (summarised in table 4), indicates that it is likely that *Xp54* is influential in the formation of masked mRNP particles containing at least three of the genes that are also analysed after morpholino treatment in fig.24 *i.e.* *cyclin B1*, *FRGY1* and *rpL1*. In view of this and the apparent effectiveness of the morpholino in inhibiting *Xp54* translation detectably over 48 hours post-injection

(fig.23), it seems unlikely that the early fluctuations seen for the mRNAs of the 3 aforementioned genes are an indirect result of fluctuations in the morpholino's efficacy as any losses of morpholino efficacy following injection would have to occur in between the time intervals shown in fig.23(b) followed by a resumption of such efficacy as to inhibit virtually all translation of the T7-Xp54 from the co-injected vector and leave virtually no visible protein at 36 post-morpholino injection (24 hours post-T7 injection, fig.23b).

By comparing the values obtained for the controls at 48 hours with the 48 hours antisense samples in fig.24 and comparing the 24 hour and 48 hour samples in fig.25 the likely influence on reduced Xp54 on the stability of various mRNAs was deduced and is summarised in table 5(a).

<u>Presumed effect of underexpression of Xp54</u>		<u>Gene mRNA produced from</u>
A.	Enhancement of mRNA stability (before maturation induction)	<i>Histone H4, rpS1, mos</i>
	Reduction of mRNA stability (before maturation induction)	<i>FRGY1</i>
B.	Enhancement of mRNA stability (after maturation induction)	<i>Histone H4, histone B4, mos, cyclin B1</i>
	Reduction of mRNA stability (after maturation induction)	<i>tcf3, β-tubulin, HDAC, FRGY1, rpL1</i>
Table 5: Influence of the anti- <i>Xp54</i> antisense morpholino on specific mRNAs analysed in this study		

Of the four genes most clearly affected by the apparent influence of the

morpholino, *histone H4*, *ribosomal protein S1* (*rpS1*), *mos* and *FRGY1*, as stated above all are thought likely to form mRNP particles with Xp54 except *mos* which was not investigated along with the genes presented in fig's 20 and 21. *Mos* mRNA however has been detected in *Xenopus* oocyte mRNP particles (Smillie and Sommerville, 2002). As a result of this association, these three genes can or probably must (for the mRNAs repressed until after maturation onset) interact with Xp54 during nuclear export in oogenesis. For *rpS1*, as described, it is thought that the overexpression of wild-type Xp54 increases nuclear export and therefore 'usage' (translation and decay) of the mRNA in the cytoplasm or that competition with a mutant Xp54 form blocks export and produces nuclear accumulation of the mRNA that leads to an increase in that gene's mRNA stability in the oocyte relative to that during expression of exogenous wild-type Xp54. For *FRGY1* however, the apparent lesser dependency on Xp54 for nuclear export means less enhancement of nuclear export when wild-type Xp54 is overexpressed and a lower blockage in nuclear export on expression of the Xp54 mutant that does not lead to such nuclear accumulation of the mRNA. As stated though, *FRGY1* mRNA that is exported to the cytoplasm does appear to decay in similarity to *histone H4* mRNA however (fig.15) rather than being stored, at least at stage 4 of oogenesis. Nevertheless, for *FRGY1* a higher mRNA stability is seen in

conditions of wild-type Xp54 overexpression relative to expression of the mutant form.

Therefore effects to the contrary for the mRNAs of these genes might well be expected when conditions of reduced Xp54 are imposed by the morpholino. As can be seen in table 5(a) *histone H4* and *rpS1* mRNA show clear indications of being stabilised as a result of the morpholino's influence whereas *FRGY1* shows a consistent reduction in stability under anti-*Xp54* morpholino conditions over the three runs of the RT-PCR analysis relative to control conditions. Comparisons between fig's 20 and 21 and fig's 24 and 25 are difficult however as in the former two the stabilities of the mRNAs are being compared between conditions of exogenous Xp54 expression and exogenous DQAD-Xp54 expression whereas in the latter two, stabilities are being compared between conditions of reduced Xp54 expression and after the injection of a relatively inert morpholino. Nevertheless it is worth considering how a reduction in Xp54 might have influenced the stabilities of certain mRNAs presented in fig's 24 and 25 in light of the apparent nature of Xp54's association with them.

Using the hypothesis outlined above attempting to explain the nature of various genes' dependencies on Xp54, a reduction in Xp54 would be thought to lead to less nuclear export of *histone H4*, *rpS1* and *FRGY1* but

there would be distinct differences between the former two genes and the latter in the manifestation of this trend. For *FRGY1*, a gene not translated until after maturation, a reduction in Xp54 would be expected to result in the formation of less mRNP particles containing its message and so potentially more mRNA not associated with masking proteins. As stated, such mechanisms are thought more probable to operate prior to masking as once masked it is understood that only dephosphorylation of FRGY2a/b occurring after maturation onset is able to cause mobilisation the mRNA onto ribosomes (Sommerville and Ladomery, 1996). Thus with reduced Xp54 and a greater amount of unmasked *FRGY1* mRNA in the oocyte, whether translation was able to take place or not, less would be able to persist and would ultimately be degraded by nuclear or cytoplasmic nucleases which may explain the apparent decrease in stability after 48 hours of anti-*Xp54* morpholino treatment (fig.24) although the stability of *FRGY1* mRNA as indicated by fig.15 argues against storage as mRNP at this stage of oogenesis.

In the case of *histone H4* and *rpS1* although it seems from the results discussed above that the mRNA for both genes associates with Xp54 in being masked in part, an increase in total stability of mRNA is observed for both. This seems contradictory to the role which Xp54 is understood to be playing in forming mRNP particles containing a fraction of the total

quantity of these mRNAs that are repressed until maturation and later. A reduction in Xp54 presence in the oocyte might be expected to result in less repression and a greater proportion of *histone H4* and *rpS1* mRNA translation and subsequent degradation but the failure to detect *histone H4* mRNA in the absence of transcription (fig.15) suggests that storage is not the goal of any Xp54 association at this stage in oogenesis. Figure 25 gives information on the proportion of such enhanced stability attributable to either oocyte cellular compartment for *histone H4* and *rpS1* mRNA and it can be seen that the enhancement is a result of greater apparent levels of mRNA present in either or both cellular compartments.

Further insight into the nature of Xp54's action on mRNA from a gene such as *rpS1* may come from looking at the effects of combined antisense morpholino and T7-*Xp54* oocyte injection (fig.26). Although *rpS1* may be stored as mRNP or at least is transported in association with Xp54, as indicated by the apparent enhanced nuclear export in fig.26, the antisense morpholino is still able to exert sufficient influence to enhance the stability of *rpS1* mRNA in the presence of simultaneous overexpression of Xp54. The situation could be as though reduced Xp54 is either allowing *rpS1* (or *histone H4*) mRNA that would normally be translated to become stabilised somehow or enabling a greater transcription rate from the relevant chromosomal loci. Of these possibilities, the former implies that Xp54

normally interacts with mRNA destined to be translated subsequent to the time of operation of whatever mechanism determines whether the mRNA is to be masked or exported for immediate translation and then promotes its degradation by some means. As described below, Xp54 is thought in fact to remain associated with mRNA that is to be bound by the Y-box proteins FRGY2a/b either to be translationally repressed and stabilised or possibly for translation. However it may be the case that it promotes translation initiation of mRNAs not masked by the Y-box proteins. If it were the case that Xp54 promoted translation of both non-masked genes and masked genes (the latter when mobilised at maturation or embryogenesis), it is still not known what mechanism determines whether a particular mRNA is to bind Xp54 and the masking proteins in such a way as to produce masking or in a way to direct that mRNA for translation. An alternative conclusion in view of previous work on Xp54's association with transcribing chromosomes could be that genes whose mRNA is stabilised, apparently by reduced Xp54, are to an extent less restricted in their transcription rate when Xp54 quantity is reduced. Xp54 in transcriptionally active oocytes localises to the RNP matrix (aggregation of various RNP proteins) surrounding chromosomal loops. The results presented in fig.14 would suggest that in stage 4 oocytes, which apply to the present results, there would still be expected to be considerable transcription, also

suggested by quantities of mRNA in the nucleus shown in fig's 20 and 21. Previous results have shown that Xp54 localises to the RNP matrix and binds indirectly or directly to transcripts emerging from the chromosomes when RNA polymerase II is operating on the DNA and dissipates by some means from this locality when RNA polymerase is inactive (Smillie and Sommerville, 2002). Thus Xp54's presence in the chromosomal loop matrix is functionally tightly linked with the activity of RNA polymerase II. Therefore one can envisage a possible scenario where the aggregation of major RNP proteins such as Xp54 limit the rate at which the enzyme can perform repeated runs of particular genes' loci and so when this RNP protein concentration is reduced, transcription is able to increase to a certain degree and transcripts can be exported to the cytoplasm to a greater extent by means other than masking proteins. However, why this phenomenon would operate more clearly on certain genes is another unknown factor. Figure 14 shows that *FRGY1* mRNA accumulates more rapidly than either *rpS1* or *histone H4* after stage 4 so the transcription rates could conceivably be higher for the latter two genes in a reduced presence of a major RNP particle protein such as Xp54 in the chromosomal loop matrix which may constitute a more significant rate-limiting factor on transcription. Thus reduced Xp54 would represent an opportunity to realize the transcriptional rate potential rather than an

effective loss of protection against cellular nucleases by assisting masking. In spite of this though, the antisense morpholino appears to stabilise *mos* mRNA (fig.24) which, along with *FRGY1*, is a gene that is translationally repressed at this stage in *Xenopus* development and thus a lower quantity of Xp54 might be expected to lead to less masking and more degradation as stated for *FRGY1* mRNA. Given the apparent constancy of the anti-*Xp54* morpholino's operation in inhibiting Xp54 translation over 48 hours (fig.23) and the ability of the morpholino to leave the quantity of certain mRNAs relatively unaffected over the same time, for example *cyclin B1* compared to *mos* mRNA in fig.24, it seems possible that the pattern of *mos* change over 48 hours is at least partly due to natural fluctuations. The reason for the increase at the 48 hour antisense sample relative to the control is unclear though and could be related to the normal degree of association of Xp54 with the locality of the *mos* chromosomal locus which the action of the antisense morpholino might reduce leading to the postulated increases in transcription suggested above for *rpS1* and *histone H4*.

In conclusion it seems that the quantity of Xp54 influences different genes to varying extents as, in a similar sense, it apparently affects the export of different mRNAs in different ways. Furthermore it may be the case that the

rate of a gene's normal transcription may affect the type of relationship it has with Xp54 in addition to the class of gene affecting the nature of the relationship as seemed to be the case with Xp54's influence on the nuclear export of a particular gene's mRNA (fig's 20 and 21). Whether a gene is destined to be translated in addition to possible storage or is a non-translated gene that is accumulating mRNA presumably for storage, may influence both the stability in the oocyte and possibly the rate of transcription from its chromosomal locus. Reducing the quantity of Xp54 seems to reveal this varying relationship to an extent but may be complicated by natural fluctuations in genes' mRNA levels. The genes that are most clearly influenced by extra or mutant Xp54 expression are not always the genes most visibly affected by reduced expression and most of the genes analysed by reduced Xp54 expression are not so affected as to make a distinction between translating and non-translating. For example it is not clear to determine from any of the results discussed to what extent Xp54 influences *histone B4* yet previous results have detected its mRNA in Xp54-containing mRNP particles of immature oocytes (Smillie and Sommerville, 2002). The differing sensitivities of the genes analysed to the quantity of cellular Xp54 may depend on the degree to which Xp54 aggregates around its chromosomal locus for association with its messages, in turn influencing such changes as nuclear mRNA

accumulation and perhaps transcription rate-determining influence of the RNP matrix.

Underexpression of Xp54 during oocyte maturation

In view of the possibility of distinctions existing between different genes' relationships with Xp54 on the basis of their translation status (*i.e.* whether they are translated before or only after maturation induction) it was deemed important to examine mRNA levels following maturation induction under conditions of reduced Xp54 expression. This distinction as it has been suggested may need to take into account factors such as different rates of transcription but it was thought that the overall picture of contrasting mRNA levels for the various genes analysed provided sufficient grounds to expect similar distinctions between mRNA levels of such genes following progesterone-induced oocyte maturation.

The significant inhibition of oocyte maturation caused by the anti-*Xp54* antisense morpholino (fig.27) manifests as both complete inhibition of maturation for at least 9 hours following progesterone stimulation (for the majority of oocytes) and a delay in the onset of maturation for the proportion of oocytes that do mature within 9 hours. This indicates that the reduction in Xp54 protein imposed by the antisense morpholino during the incubation time before progesterone stimulation is sufficient to impair the

maturation process and that Xp54 continues to have a role during maturation which is crucial to the physiological changes occurring in the oocyte. This role, in the absence of transcription, is thought to be related to translation initiation facilitating the production of proteins from masked maternal mRNA molecules accumulated as mRNP over the course of oogenesis. It seems reasonable to conclude that Xp54 is probably associated with the mRNA from one or more than one gene which is untranslated before maturation and which is essential to or facilitates the progress of maturation. Such genes might include *mos* or *cyclin B1* whose mRNAs are components of Xp54-containing mRNP particles (Sommerville and Smillie, 2002) and, as this study has indicated, are influenced in their export and stability by Xp54.

The discernible influence of Xp54 on the net stability of the range of genes analysed in fig.28 at 9 hours when the potency of the effect on maturation of the antisense's reduction of Xp54 quantity is clearest is summarised in table 5. The distinction between genes translating during oogenesis and those translated only after maturation is more striking than in fig's 24 and 25. Of the mRNAs from translating genes both *histone deacetylase* (*HDAC*) and *rpL1* show apparent destabilisation by the antisense not clearly seen before maturation (fig.24 and 25). This is in addition to the destabilisation of *pcf3* (subject to possible error), *beta-tubulin* and, as in

immature oocytes, of *FRGY1*. The shift to a destabilising influence seen with the antisense morpholino is thought to represent the shift of Xp54 from a restricting influence on transcribing alleles to solely a regulator of translation. A reduction in oocyte Xp54 translation caused by the anti-Xp54 morpholino in the stage 6 oocytes (during the incubation period before maturation stimulation) may not be sufficient to allow any detectable increase in transcription of various genes to occur, assuming Xp54 has some restricting influence. However the blocking of Xp54 translation by the morpholino may be sufficient to reduce the amount of Xp54 incorporated into mRNP particles during this period and therefore the quantity of mRNA stored with Xp54 relative to the control-injected samples. In support of this, fig.14 indicates that mRNA levels per oocyte are highest at stage 6 following consecutive increases during oogenesis although it is not known what fraction of this represents transcripts that are destined to be incorporated in mRNP or destined to be translated. Consequently, at 9 hours post progesterone stimulation when the mobilisation of stored mRNA is likely to be well underway and unmasked transcripts residual from the onset of maturation have possibly degraded, the discrepancy is most visible. As stated already, whether Xp54 is essential for masked mRNA export or not the results of this study indicate it is required for efficient export of, potentially, masked mRNA and

therefore a reduction would be expected to leave mRNA to be either degraded or perhaps be directed into an export route destined for immediate translation. It is conceivable however that mRNA might be directed, at a lower degree of efficiency, into export for masking without Xp54 although for a net destabilisation to occur as seen here it is thought less conceivable that such export would be significant. Consequently at this stage in the development of the oocyte, it is thought that the effect of the antisense morpholino seen with genes such as *pcf3* reflects the reduced capacity of the oocyte to store the mRNAs which become, in effect, destabilised (table 5; fig.28). Though the effect of the morpholino on *FRGY1* mRNA before 48 hours may be a net destabilisation in stage 4 oocytes (fig.24) it may be the case that there exists a transcription rate in stage 6 oocytes on which the morpholino, after the incubation period of around 15 hours (the 3 hour sample in fig.28), is able to relieve the limiting effect of Xp54 on transcription hypothesized in this study whose consequences — *i.e.* a greater quantity of mRNA — are still evident at 3 hours post-maturation induction. At 9 hours the reduced-storage effect induced by the morpholino seen as with the other genes whose mRNA is destabilised might be the case for *FRGY1*. Although fig.15 as stated above does not suggest significant storage of *FRGY1* mRNA as mRNP during stage 4 of oogenesis, in contrast to *histone B4*, more storage might occur

subsequently.

Of the other genes analysed that do not translate in immature oocytes, all show stabilisation at 9 hours, *i.e.* *histone B4*, *cyclin B1* and *mos*. For this to occur at this time during maturation, it seems less likely that any change in mRNA level relative to the control would be due to reduced Xp54 translation and availability for mRNP incorporation during the incubation period with the morpholino as if this were the case then destabilisation would be more likely as seen with genes such as *rpL1* and *pcf3*. One possibility is to consider that Xp54 unassociated with mRNP particles continues to perform an important role during maturation and subsequently and that the inhibition of this role by morpholino-impeded Xp54 translation during maturation results in a net stabilisation of the 3 mRNAs in the lower half of fig.28. Despite the decline of Xp54 mRNA levels after stage 2 oocytes, it is nevertheless detectable into embryogenesis and more importantly the protein continues to be clearly expressed after the blastula stage of embryogenesis (Ladomery *et al.*, 1997), *i.e.* longer than FRGY2a/b, suggesting that this may represent translation of stored Xp54 mRNA in addition to mRNP-bound Xp54 that is being detected. It is therefore thought possible that for these genes free Xp54 may be contributing to the efficiency of translation (presumably in translation initiation as stated) and in the absence of a normal quantity of available Xp54, as in the antisense

morpholino-treated samples, less translation and subsequent degradation of their mRNAs occurs effectively causing their stabilisation. It may be the case that the mRNAs which are apparently destabilised by the antisense morpholino in fig.28 are similarly affected by the reduction in available Xp54 but the crucial difference could be that these genes, as described above, are more affected by the reduced Xp54 during the incubation period before maturation induction in representing genes that are (with the exception of *FRGY1*) continually needed through oogenesis and thus undergoing ongoing translation (and in part stored as mRNP until the onset of maturation) in contrast to the three genes in the lower part of fig.28 whose mobilisation from mRNP would be expected to be much greater at the onset of maturation. There may well be greater mobilisation of *mos* and *cyclin B1* mRNA given the vital roles of their proteins in oocyte meiotic maturation. In chromatin remodelling, *histone B4* is also understood to have a significant role to play during maturation (Hock *et al.*, 1993). Thus these genes might be particularly affected by reduced cellular Xp54 (assuming free Xp54 plays a role in the efficiency of translation).

Histone H4 mRNA levels also indicate stabilisation by the morpholino as in immature oocytes and perhaps the impediment on its transcription by Xp54 relative to morpholino-treated samples is such that the increase in its transcription resulting from reduced Xp54 expression is still evident at the

9 hour time interval or the situation could be that *histone H4* mRNA undergoes a notable translation burst that normal levels of free Xp54 might contribute to thus leading to, normally, more degradation of the message. It may be the case that *histone H4* and *FRGY1* are exceptions to the apparent relationships existing between Xp54 and genes translating before or after maturation onset which are difficult to explain but a recognisable general distinction between the genes based on their translation status is worthy of note.

Interaction of other mRNP proteins with mRNA and Xp54

Cumulative evidence from studies on the major oocyte mRNP proteins has given rise to maskosome theory of such proteins cooperating to form translationally repressed mRNA-containing particles (Sommerville, 1999). The masking proteins FRGY2a and FRGY2b experience very similar distribution and localisation changes during oogenesis as Xp54 and the Y-box proteins can be isolated in the same fractions. That these proteins are associated with mRNAs in a relationship concerned with translational control is reinforced by the results presented in fig.29 which is summarised in table 6. It is shown that the presumed reduction in FRGY2a/b levels in immature oocytes produces clear changes in the net stability of specific mRNAs from which some distinctions can be made based on translational

status. The differing influence of FRGY2a/b according to mRNAs may relate to the idea of a balance existing in the potential of FRGY2a/b to promote or repress translation depending on its quantity as exemplified by low quantities of the Y-box masking protein p50 in rabbit somatic cells being present on translating mRNA and low quantities enhancing *in vitro* translation (Evdokimova *et al.*, 1998). Thus for the genes in fig.29 that are masked and do not therefore translate in the immature oocyte, *mos* and *FRGY1* (which incidentally may perform in somatic *Xenopus* cells the same role as p50), it is possible that normally greater quantities of FRGY2a/b are bound within the mRNP particle to the mRNA thereby repressing translation. A reduction in FRGY2a/b levels might therefore account for the destabilisation of their mRNA in releasing to a certain extent the block on translation and subsequent degradation proceeding. However this is difficult to corroborate with the results in fig.15 which suggest that neither *mos* nor *FRGY1* mRNA is stored to any significant extent at this stage in oogenesis. Thus whatever the nature of any interaction between these mRNAs and FRGY2a/b protein it is possible it promotes stability presumably by cooperating with other mechanisms to repress translation.

For the translating genes *rpL1* and *pcf3*, it is possible that normally a substantial proportion of transcripts that are not being masked for use

during maturation/embryogenesis are associated with low quantities of FRGY2a/b which is somehow promoting their translation. Thus in the presence of reduced FRGY2a/b levels the rate of translation and subsequent degradation of these mRNAs is accordingly lowered accounting for the corresponding increase in their stability relative to control conditions. In spite of this distinction between translating and non-translating genes, *cyclin B1* — a gene translated for a vital role during maturation and not before — shows the apparently characteristic stabilisation of translating genes in fig.29. In view of the proposed possible mechanics of the masking process outlined in this study on the basis of the results presented, it is concluded that the only conceivable means by which this might occur is if there existed differences in the quantity of masking proteins that accumulate around and then associate with different transcripts. If *cyclin B1* represented a gene whose transcripts were more extensively associated with FRGY2a/b or its transcription rate was higher than the other masked genes at this stage of oogenesis then the greater aggregation of the masking proteins at its locus might impose a limit on the rate of repeated transits by RNA polymerase in transcribing *cyclin B1* as proposed above for the effect of the anti-*Xp54* antisense morpholino on certain genes. The relief of such a greater impediment on transcription compared to *mos* and *FRGY1* might enable the realization of or the

progress towards the transcription potential of *cyclin B1* more notably and consequently increase the quantity of *cyclin B1* transcript to a greater extent relative to the control. Figure 14 indicates that *cyclin B1* does show a rapid increase in its mRNA accumulation per oocyte between stages 4 and 6 of oogenesis that is significantly more than *mos* or *FRGY1* although whether *FRGY2a/b* levels would impose a restriction on yet a further increase in transcription remains speculative.

<u>Presumed effect of underexpression of <i>FRGY2</i></u>	<u>Gene mRNA produced from</u>
Enhancement of mRNA stability	<i>tcf3</i> , <i>rpL1</i> , <i>cyclin B1</i>
Reduction of mRNA stability	<i>mos</i> , <i>FRGY1</i>
Table 6: Influence of anti- <i>FRGY2</i> morpholino on specific mRNAs analysed in this study	

A picture of the nature and extent of Xp54 and FRGY2a/b's interaction with mRNA can be derived from the investigations into specific mRNA quantities but this understanding of the mechanics of Xp54 and FRGY2a/b in regulating translation can be supported further by examining protein levels under conditions when Xp54 or FRGY2a/b levels have been perturbed. Indeed the effect on general protein levels indicates that the role Xp54 plays in immature oocytes is in limiting significant quantities of translation as when the anti-*Xp54* morpholino is used to reduce cellular Xp54, rates of protein synthesis increase (fig.30) across a broad range of the most abundant cellular proteins. In light of what is known about Xp54, this limiting is thought to be the action of Xp54 in contributing to the formation of mRNP particles.

With less Xp54 in the oocyte more mRNA is thought to be directed into routes destined for translation which in turn would suggest that most mRNAs in the oocyte would follow the example of *FRGY1* in immature oocytes (fig.24) in being destabilised by translation and/or degradation when Xp54 levels are reduced. However, genes could also be increasing their transcription rates as proposed for *histone H4* (fig.25) for the 24 hours prior to addition of alpha-amanitin to the oocyte buffer which could also contribute to the enhanced translation rates seen in fig.30 assuming a significant proportion of the enhanced amount of mRNA produced is

directed to nuclear export routes for translation in the cytoplasm as opposed to becoming masked. It is unclear what proportion of the enhanced translation would be accounted for by any enhancement in unmasked transcript production and if this would be significant. Also, inhibition of FRGY2a/b translation with the anti-*FRGY2* morpholino has the reverse effect of reducing rates of general protein synthesis in the oocyte whereas a similar enhancement of transcription as proposed for the anti-*Xp54* morpholino would be thought to work contrary to the apparent inhibition of translation rates as seen in fig.30. Either way, it is thought reasonable to conclude that with a reduction in cellular Xp54, less mRNAs become masked and presumably, in effect, stabilised for the period of investigation.

Such differences in translation occurring in the 24 hours after inhibition of further transcription in the autoradiograph of fig.31 strongly suggests that more mRNA is freely available to translate following reduction of cellular Xp54 levels. The reduced protein synthesis rate (fig.30) resulting from lowered cellular FRGY2a/b levels might suggest that FRGY2a/b normally is somehow stabilising most oocyte mRNAs for translation but in view of FRGY2a/b's masking function and the stage of the oocyte under investigation when masked mRNAs are not being mobilised onto polysomes (and hence not being unmasked and translated) this is thought

unlikely. It is concluded that more probably, as stated for specific genes in fig.29, FRGY2a/b normally has a role in promoting translation of most oocyte mRNAs that produce proteins in any significant quantity *i.e.* those proteins seen in the fig.31 autoradiograph. Thus it is envisaged that low quantities of FRGY2a/b are bound to most mRNAs and when levels of the protein are reduced this promotion is less prevalent with a corresponding reduction in translation of available mRNA as witnessed in fig.31. It follows that though there would be expected to be destabilisation to a degree (possibly after inappropriate translation) of normally masked messages, any translation occurring from these messages is not sufficient to negate the general inhibition in translation observed.

An increase and decrease in new protein synthesis relative to uninjected oocytes by the anti-*Xp54* and anti-*FRGY2* morpholinos respectively is indicated by fig.32. The possibility that the changes in the total proteins synthesised following morpholino addition relative to the control in fig.31 are more related to any influence *Xp54* or *FRGY2a/b* may have on protein rather than mRNA stability is made more unlikely by the more direct visualization of new protein synthesis made possible by the extraction of polypeptides associated with poly(A)+ material presented in fig.32. This suggests that both proteins operate on the mRNA rather than as post-translational mechanisms.

Whereas the binding of large quantities of FRGY2a/b to mRNA is perhaps easier to associate with its consequence of repressing translation of the message, how might a lower quantity promote translation? One possibility is described by Mansfield *et al.* (2002) for Yps, a Y-box protein found in mRNP particles with the *Drosophila* homologue of Xp54, Me31b. The accumulation of Yps at the posterior pole with a specific mRNA results in translation of the mRNA despite Yps normally repressing it. Mansfield *et al.* suggest that, based on work with the aforementioned p50 (rabbit Y-box protein), Yps may be able to bind actin microfilaments of the oocyte cytoskeleton which could modify the activity of Yps to somehow promote translation for example by immobilising an mRNA in a region where factors which altered such activity are concentrated.

A more direct explanation is suggested by the action of CspA, a protein involved in the cold-shock response of *E.coli*. Its nucleic acid-binding domain named the cold shock domain as described in Introduction is found in many proteins in vertebrates and invertebrates including the Y-box proteins. During low temperature growth conditions, CspA maintains the single-stranded structure of mRNA molecules thereby contributing towards their efficient translation (Jiang *et al.*, 1997).

Regardless of the specific means of translation promotion by lower quantities of FRGY2a/b, at higher concentrations it is perhaps not too

difficult to imagine the scale of binding becoming inhibitory to the process of translation and thus negating any promoting action the protein may have owing to the physical impediment posed by its abundance.

Xp54 and FRGY2 as multifunctional proteins

That Xp54 and FRGY2a/b cooperate in *Xenopus* oocyte mRNP particles is well established but it is concluded that there is evidence here that suggests a distinction in the nature of this cooperation according to the translational status of the mRNAs involved in this cooperation that arises directly out of the necessity to store, or in certain cases repress, a fraction of the total transcripts produced during oogenesis for later stages when transcription is inhibited and specific proteins are required immediately. The results here indicate that this distinction is mainly observable between translating and non-translating mRNAs as the repressed mRNAs become mobilised during maturation.

It seems clear that for the majority of genes normally producing abundant proteins during oogenesis, Xp54 represses translation in part, presumably in storing their respective mRNAs as mRNP particles and FRGY2a/b is able to contribute to this storage and translation repression whilst also being able to promote translation of a significant proportion of the same genes. However the influence of Xp54, as a result of its status as a major

mRNP protein, may be that it imposes a impediment on transcription for certain genes whilst merely determining the amount that can be stored as mRNP for others.

When Xp54 levels are lowered the resulting reduced storage is seen in the form of greater general translation of proteins in the oocyte and in mature oocytes in the form of a reduced quantity of mRNAs available to translate, for specific translating genes. FRGY2a/b's influence is also seen when its levels are reduced but as a promoter of translation of genes that translate before maturation in the form of reduced general protein synthesis in the oocyte and in the greater quantity of mRNA for specific translating genes. The dual functioning of FRGY2a/b is exhibited by non-translating, repressed genes (such as *mos* and *FRGY1*) whose mRNAs, are concluded to be less likely subject to the translation-promoting influence of FRGY2a/b but its translation-repression/storage influence only and consequently exhibit lower quantities of mRNA when FRGY2a/b levels are reduced. It should be noted though that the storage of *mos* and *FRGY1* mRNA seems to be very limited at stage 4 of oogenesis relative to *histone B4*. A second function is also possible for Xp54 with such genes in mature oocytes as stated above, which may not only reveal the role of Xp54 within mRNP particles of all genes that are being activated but a role for free cytoplasmic Xp54. It is possible that such Xp54 is contributing to the translation of

masked mRNAs that are becoming mobilised and this particularly affects genes which do not translate before maturation and have essential functions during maturation which may necessitate greater overall translation of their mRNAs.

Indeed the possibility of Xp54 or its homologues being capable of participating in apparently diverse activities in the cytoplasm is strengthened by recent work indicating a role for the *Saccharomyces cerevisiae* homologue, Dhh1p, in processing or degradation bodies ('P-bodies') — mRNA-protein aggregations in yeast that are thought to contribute to deadenylation-dependent mRNA decay (Sheth and Parker, 2003). As described in Introduction, deadenylation-dependent decay is understood to be the predominant mRNA decay pathway in yeast and within these structures Dhh1p is understood to assist the functioning of the decapping enzyme Dcp1. Sheth and Parker also suggest that Dhh1p might also assist in translation control in removing mRNA not required for translation as when translation initiation is inhibited an increase in decapping results, the reverse of which is the case when cycloheximide is used to 'freeze' mRNAs in their interaction with ribosomes. Thus as Dhh1p seems to be involved in mRNA degradation, Xp54 seems to be involved in both translation repression and promotion of translation initiation.

Therefore, the results underline the combined importance, in more than

one function, of Xp54 and FRGY2a/b in translational control throughout oogenesis but also indicate the different significance of these actions according to the translational status of the gene in question.

RAP55

The mRNP component RAP55 (predicted mass corrected in the databanks as 51.9KDa) identified in the salamander *Pleurodeles* by Lieb *et al.* (1998) is a candidate for an Xp54 companion protein and like Xp54 and the Y-box masking proteins was thought likely to represent one of the four major *Xenopus* mRNP particle components identified by Darnborough and Ford (1981) — 'RNP2'/p52. RAP55 has a reported lack of ability to be crosslinked to mRNA by UV-treatment (Lieb *et al.*, 1998) and, like Xp54, there is a gradual decline in its quantity during early embryogenesis. The former suggests that RAP55 is bound to mRNP particles by protein-protein bonds although the presence of two RGG-boxes, found commonly in RNA-binding proteins, suggests that assistance in the mRNA-binding by another mRNP component may be part of the protein's function. It has not been certain whether the component with which RAP55 interacts is Xp54 and if this is for the purpose of maintaining mRNA in a stored state until its mobilisation for example after fertilisation when both proteins decline. The rationale of the investigations in the present study therefore was to

look for evidence of any interactions between Xp54 and RNP2 and to determine if RNP2 in *Xenopus* was an orthologue of RAP55 identified in *Pleurodeles*.

The evidence for RNA-Xp54 crosslinking in figure 33 does not refute the reported lack of ability of Xp54 to become substantially UV-crosslinked to mRNA (Ladomery *et al.*, 1997) but rather supports the notion that Xp54 interacts more extensively with another protein component or components rather than mRNA within mRNP. The evidence in figure 33 strongly indicates that such a component is RNP2/p52 as judged by their coincidence on the immunoblot following crosslinking, an interaction which does not seem to depend upon RNA. In figure 34 however, the evidence for crosslinking of Xp54 to an unidentified mRNP component is indirect coming from the reduced quantity of Xp54 or T7-Xp54 on UV-treatment. The presence of a higher band (suspected of representing protein-protein crosslinking rather than RNA interaction) within the Xp54 immunoblot of fig.34 would have provided a basis for comparison to the RNP2 immunoblot in fig.35 but protein-protein complexing may have produced a molecule that was too large to transfer from the SDS-PAGE gel. However, that the co-expression of the first *RAP55*-candidate (XRAPA) from the pCMV vector detracts further from the Xp54/T7-Xp54 bands (fig.34) is further indirect support for existence of a complex that

results from Xp54-RAP55 crosslinking and thus close proximity within mRNP.

The anti-RNP2 antibodies detect a 52KDa protein (originally suspected as a RAP55 orthologue candidate) as expected and like Xp54 seems to be capable of being crosslinked to an unidentified mRNP component or components forming a complex not seen on the immunoblot as evidenced by a reduction in band intensity on UV-treatment (fig.35). This crosslinking is apparently facilitated by the presence of RNA which noticeably detracts from the 52KDa band intensity in contrast to T7-Xp54 in figure 34 which remains almost unaffected on crosslinking.

Therefore the evidence for a close proximity between Xp54 and any RAP55 orthologue candidate such as XRAPA, in contrast to RNP2 from poly(A)+ material (fig.33) remains circumstantial. The sequencing and alignment (table 3, Appendix B+C) of cloned *Xenopus* cDNAs for XRAPA and for a second *RAP55*-like clone however have made the situation clearer regarding the identity of p52 and XRAPA microinjected in fig's 34-35.

With a much higher protein sequence similarity and identity and similar structural arrangement to RAP55, XRAPA would appear to be the most likely candidate for a RAP55 orthologue rather than XRAPB. The identity of the XRAPA protein sequence and near identity of the cDNA sequence

to those of Q8AVJ2 (whose mRNA has been detected in *Xenopus* embryos) might suggest either that the genes are in fact the same with discrepancies arising from the two separate cDNA sequencing procedures or that they represent homologous genes expressed from either chromosome set of this tetraploid organism. The identity of the translated sequence of XRAPB with the unpublished peptides sequences from p52 extracted from SDS-PAGE gels suggests that XRAPB represents this protein and is furthermore an orthologue of the novel human protein Q9BX40 (UNIPROT accession no.) with which it has a high similarity and identity. In turn this indicates that the 52KDa protein that was seen and apparently crosslinked to an unidentified factor or factors in fig.35 represents endogenous p52 rather than XRAPA that, like Xp54 (fig.34), can apparently become assembled into newly-formed T7-Xp54 containing particles. In view of the relatively lower similarity and identity between XRAPA and XRAPB compared to Q9BX40 and RAP55 (Q9YH12) respectively it is perhaps not surprising that the anti-RNP2 antibody failed to detect any XRAPA expressed from the microinjected pCMV expression vector.

However, a higher molecular weight complex formed as a result of UV-induced crosslinking and likely to contain Xp54 as well is not seen with p52 in fig.35 as it is in fig.33. It is not clear why there should be this

difference. The protein extracted in fig's 34 and 35 represent a more specific extraction method using immunoprecipitation rather than extraction of poly(A)+ material. Whether a greater selection for mRNA in fig.33 enables the crosslinking of p52 and Xp54 is not certain nor if some other factor becomes involved later in oogenesis (extractions were from later in oogenesis in fig.34 and 35) that makes the any crosslinking involving Xp54 and p52 more complex.

The significance of the sequencing and alignments then is that p52 and the likely *Xenopus* RAP55 orthologue, XRAPA, seem to be separate, but related proteins. Both XRAPA and XRAPB were identified as clones similar to the cDNA for *RAP55* and thus following alignment seem to be related (table 3) but XRAPB appears to have diverged further from any common ancestor. The situation of determining the identity of these clones was confused by the predicted molecular masses of their proteins and by the apparent masses of RNP2 and RAP55. The apparent mass of 52KDa (RNP2) is closer to the predicted mass of 51.3KDa for XRAPA rather than the 45.7KDa for XRAPB. The apparent mass of RAP55 in *Xenopus* and *Pleurodeles* is 68KDa (Lieb *et al.*, 1998) which is also closer to the predicted mass of XRAPA. In view of the such a discrepancy between predicted and apparent mass in RAP55 and the similar sequence and structural arrangement with XRAPA as described in Results it is likely that

XRAPA would migrate in SDS-PAGE with a similar inconsistency although this remains to be confirmed. This makes speculation possible that the 68KDa protein isolated from mRNP by Lodomery *et al.* (1997) and which, along with Xp54, could be photo-crosslinked to ATP represents XRAPA, the *Xenopus* RAP55 orthologue.

Similarly, the high concentration of inward facing hydrophobic prolines at the N-terminus of XRAPB and the large number of basic arginines — as in RAP55 and XRAPA — might be expected to cause XRAPB to migrate more slowly than its predicted mass would imply. This of course is supported by the XRAPB sequence identity to the sequenced p52 peptides (J.Sommerville, unpublished) as stated above.

Given that XRAPA and XRAPB are calculated to have 57% similarity to each other and similarity, to a varying extent, with RAP55 (Appendix C) another possibility is that both proteins represent isoforms that perform similar functions but at different stages of *Xenopus* development. In *Pleurodeles* at least RAP55 is thought to decrease during early embryogenesis, more rapidly than Xp54, although may be converted to another form subsequently (Lieb *et al.*, 1998). Initial experiments indicate that *XRAPA* mRNA cannot be detected by RT-PCR after stage 4 of oogenesis in contrast to *XRAPB* which is detected until at least stage 6 (J.Sommerville, unpublished) so if translation corresponds to this mRNA

abundance then XRAPB could take over as the main effector of XRAPA's functioning after the onset of late oogenesis. However *XRAPA* and *XRAPB* do not appear to be oocyte-specific mRNAs as ESTs with sequences overlapping either clone have been located in somatic tissues and can be found in the databank collection of *RAP55*-like cDNAs *e.g.* BG731378 in lung tissue (table 3). Also the mRNA for Q8AVJ2, whose primary protein sequence is identical to XRAPA, can be detected in embryos making any such relationship between XRAPA and XRAPB more complex.

Summary

- For genes producing mRNAs representing all kinds of translational status there appears to be a consistent accumulation of their mRNAs within individual oocytes suggesting transcription of these genes continues until the end of oogenesis.
- A distinction in stability seems to exist between mRNAs in that some mRNAs don't seem to have a residual presence in the absence of transcription in stage 4 of oogenesis. However, at the same stage of oogenesis the same mRNAs were capable of associating with T7-Xp54. Also, it is found that a reduction in Xp54 levels during this stage of oogenesis appears to lead to a widespread enhancement in translation levels which would nevertheless suggest a general role for Xp54 in mRNA masking and potentially stabilisation.
- The DEAD motif contributes to the efficient nuclear import of Xp54 in transcriptionally active oocytes.
- Overexpression of Xp54 enhances nuclear export of mRNA both from genes destined to be translated and from those repressed until maturation possibly by hastening the export of the fraction of a gene's mRNA that is exported as mRNP. Exceptions exist with certain genes whereby Xp54 availability does not impose such a limiting factor to the export efficiency.
- It is anticipated that for the mRNA of genes which appear to have a

greater dependency on functional Xp54 for efficient nuclear export, such as *rpLL*, expression of exogenous DQAD-Xp54 results in a net stabilisation of the particular mRNA relative to oocytes with overexpressed wild-type Xp54 owing to a 'bottleneck' effect with accumulation of the message during the inhibited overall mRNA nuclear export in DQAD-Xp54 injected oocytes and more degradation of mRNA following translation in the cytoplasm of wild-type Xp54-injected oocytes.

- The anti-Xp54 antisense morpholino is able to significantly inhibit translation from an exogenous target mRNA sequence for at least 36 hours by blocking Xp54 mRNA from translation and degradation. It is also able to impact notably on the total pool of endogenous Xp54 protein after several hours which leads to a decline in total oocyte Xp54 levels owing to the limited stability of Xp54. That is, total levels of Xp54 in the oocyte decline unless fresh translation is occurring.

- The anti-Xp54 antisense morpholino is able to effect a net reduction in stability of *FRGY1* mRNA, a non-translating gene but paradoxically increase the mRNA stability of a translating gene such as *ribosomal protein S1*, in immature oocytes. These effects could be related to Xp54 involvement in mRNA storage and influence on the transcription rate of certain translating genes. Additionally, Xp54 might act as a promoter of translation initiation for non-masked mRNAs, a role that would be

repressed in mRNP particles.

- It is possible that Xp54 and FRGY2a/b as major components of the mRNP chromosomal loop matrix impose a rate-limiting factor upon transcription rate for certain genes in a mechanism related to the efficiency of RNP polymerase II.
- Xp54 has a crucial role in the physiological changes occurring during maturation such that a reduction in cellular levels of the protein leads to a significant postponement of maturation's progress.
- The apparent effect of reduced Xp54 levels in enhancing the stability of the mRNA of genes that are only activated during maturation and are vital to the process may reflect the action of free Xp54, perhaps translated after maturation induction from stored mRNA, which contributes to the efficiency of translation (presumably initiation) of such critical genes that would be expected to be undergoing significant mobilisation at this time. In any case a distinction in the effect of Xp54 on net mRNA stability is notable between previously (before maturation onset) non-translating genes and translating genes at the time when GVBD is most prevalent in oocytes.
- The influence of FRGY2a/b also apparently differs between genes translating in immature oocytes and those repressed. Its influence on net mRNA stability may depend on the quantity bound to most of a gene's

transcripts determining whether translation is repressed or promoted thereby revealing its dual functioning nature.

- It is likely that both Xp54 and FRGY2a/b operate at the mRNA level rather than the protein level in repressing or promoting a substantial fraction of cellular translation. Before maturation, the major effect of Xp54's action is to inhibit the translation of most abundant oocyte proteins presumably by mRNP storage of mRNA whereas FRGY2a/b has the effect of promoting general translation in the oocyte suggesting that the largest proportion of most translating mRNAs are bound by FRGY2a/b at levels below those required to repress translation.
- In questioning the effect of Xp54 and FRGY2a/b on specific mRNAs, it is necessary to consider the translational status of the gene in question and how this status might change during oogenesis. However both proteins are thought to interact with at least a part of the total transcripts of most genes throughout oogenesis.
- On investigating the major mRNP component mRNP2 and its relationship to the *Pleurodeles* protein RAP55, clones were identified from the EST databanks with similarity to RAP55 cDNA and sequenced. One clone, termed XRAPA shows the greatest similarity to RAP55 in protein sequence and structural arrangement and may have been visualized before RAP55 as a 68KDa protein in *Xenopus*. Indirect evidence for a close

association of this protein with Xp54 was found. Another clone, termed XRAPB, has regions of protein sequence identical to unpublished stretches of sequence generated from p52 extracted from SDS-PAGE gels. Both XRAPA and XRAPB may have originally descended from a common ancestor shared with *Pleurodeles* RAP55 but XRAPB seems to have diverged further in sequence.

Further work

In view of the involvement of Xp54 in the efficient export of a substantial proportion of most transcripts analysed in this study it would be useful to investigate more comprehensively the nuclear export of a range of mRNAs in oocytes injected with the *Xp54* morpholino. Such oocytes could be subject to reduced storage of mRNA as mRNP particles as a result of reduced Xp54 presence in the mRNP matrix and might therefore be assumed to exhibit reduced nuclear export of certain mRNAs. The results for *histone H4* in figure 25 seem to contradict this which might indicate merely that more transcripts are being directed into export for immediate translation rather than storage. The increase in net mRNA stability following antisense treatment as stated suggests that either transcription rates may have increased as well or free Xp54 is serving as a promoter for translation initiation of non-masked mRNAs in immature oocytes. Thus by analysing a range of translating and non-translating genes in immature oocytes as in fig.25 it should be possible both to gain an indication of the proportion of a gene's transcripts exported from the nucleus whose efficiency depends on Xp54 and to see more comprehensively the consequences of reducing Xp54 levels on nuclear mRNA export and mRNA stability. In particular, the lack of storage of most of the mRNAs analysed in fig.15 raises the question of what influence, if any, Xp54 has

on long term storage of mRNA as mRNP.

Investigating whether FRGY2a/b has such an effect on transcription would be more difficult to determine by this approach as net mRNA stability is presumably affected by FRGY2a/b's apparent influence in promoting translation of a transcript.

Overexpression of Xp54, in addition to the effects described, may impede transcription for certain genes. Thus, expanding the experiment to include analyses of other translating genes may indicate if this phenomenon is shared more widely. To investigate more thoroughly the influence of FRGY2a/b on specific mRNAs, it will be necessary to clone the gene and construct an appropriate expression vector for oocyte overexpression experiments.

It seems clear that Xp54 interacts with a substantial fraction of mRNAs as judged by the wide-ranging translation enhancement when Xp54 levels are lowered. Thus it is possible that non-translating mRNAs and a proportion of those mRNAs that do translate in immature oocytes may both interact with Xp54 in such a way as to restrict translation. In order to gain the most accurate indication of its significance in the oocyte however, it will be first necessary to understand better how Xp54 is able, as indicated by the results here, to influence differentially the stability of mRNAs and yet restrict the translation of a wide range of mRNAs producing abundant

proteins. There would then be a better basis for understanding how Xp54 fits into the currently understood scheme of mRNA export and storage or translation.

The functions of the *Xenopus* RAP55 orthologue, XRAPA, and the related p52 candidate protein XRAPB remain open to speculation as do the exact nature of any interactions with Xp54. It is clear from the results presented in this report that p52 and RAP55 need to be regarded as separate proteins but given the similarity between the proteins and cDNA of XRAPA and XRAPB and the likely coincidence in their subcellular localization in mRNP it would probably be wise to undertake investigations initially on both genes in concert. Structural analysis of RAP55 has revealed a proline-rich region constituting potential hydrophobic-interaction protein-protein binding sites, RGG box motifs characteristic of mRNA-binding proteins and potential phosphorylation sites for protein kinase C which is a component of mRNP proteins (Lieb *et al.*, 1998). Equivalent regions of XRAPA are highlighted in this report. However, before investigating such specific aspects concerning the regulation and activity of XRAPA/XRAPB it may be more productive to first look at the nature of the genes' expression in the oocyte for example the expression of XRAPA mRNA and protein during oogenesis. Then, changes in other mRNA and protein levels following perturbations in cellular levels of the XRAPA or XRAPB could

be examined, as has been investigated for Xp54 in this study, to determine if these proteins do indeed contribute to translational regulation. It might then be possible to combine such perturbations with other treatments such as kinase inhibition or even Xp54 expression inhibition to begin to gain information about XRAPA/XRAPB function from the factors in the cell which they can affect or be affected by.

Appendix A: Genes examined by RT-PCR

Gene	Predicted RT-PCR fragment size	Start & end within cDNA	Primer Sequences (upper is sense primer, lower is antisense primer)	Protein M _r (Da)	Function	Translated before oocyte maturation?	Databank accession number (SWISS-PROT/EMBL)
Ribosomal protein L1	240	2343-2800	5'-GTGCTGAATCGTGGGAACTGGTC-3' 5'-GCAGGAAGGGCTGAGGCGGCCAG-3'	44935	Structural ribosomal protein	✓	P08429
Ribosomal protein S1	367	7162-9859	5'-CATCATGGAGAGCGGAGCCAAGGGTTG 5'-CTGTGGCAACAGGCTGTGGCATGACG-3'	26977	Structural ribosomal protein	✓	P47835
Histone B4	150	294-415	5'-CAGTTGCTGCACCTGAGGGAGCC-3' 5'-CGACAGGGTTGGGGGATGTG-3'	29310	Linker protein involved in chromatin compactness	✗	P15308
Mos	420/541	365-906	5'-GAGGGGAGACGGTGGCGCTGAAGAAG-3' 5'-GAGGGTGATAGCAAACGAGTAGATGTCGG-3'	36162	Key regulator of meiotic progression during oogenesis	✗	P12965
CyclinB1	400	156-568	5'-GGCTGGAAGAGGGTTGTTG-3' 5'-GAAGGTAGTTTTGCTGACTGC-3'	44673	Component of maturation-promoting factor which drives oocyte maturation	✗	P13350
FRGY1	288	186-473	5'-GCAGCCAGACGCATTGGAGGGCAAG-3' 5'-GGACCAGTTACATTAGCTGCCTCTGCAC-3'	34633	Somatic cell equivalent of oocyte mRNA packaging protein, FRGY2	✗	P21573
Histone Deacetylase	369	678-1047	5'-GCCTTACGGGATGGGATTGACGAT-3' 5'-GTTGGATGGGCTGATGTGAAGCTTG-3'	54747	Enzyme modulating chromatin structure	✓	Q91695
β-tubulin	292	357-649	5'-CCCCAACAAACGTTAAGACCGCAGTC-3' 5'-CCTCCTCTTCCCCTTCTCAAACACTC-3'	49724	Microtubule structural protein	✓	P13602
xTcf3	541	1064-1418	5'-GAGGGCTAAGGTAGTGGCAGAGTGAC-3' 5'-GGAGAAGCCAGAGCAGCAGACGGAG-3'	62298 (mouse)	Transcriptional regulator	✓	P70062
p54	370	646-1016	5'-CCTACCTCATTCCCTTACTTGAACGGCTAGACC-3' 5'-CAGCAGTTTGTCTGCCTCATCCAACACAATCATC-3'	54087	Major component of oocyte mRNP particles. RNA helicase with possible roles in mRNP assembly and translation.	✓	P54824
FRGY2a	305	282-587	5'-GCAGTGTGGGTGATGGTGAGACGGTGGAG-3' 5'-TCCCTGGTTCGTGCTGTTATTGGGTCTGGG-3'	37202	Major component of oocyte mRNP particles. mRNA masking protein also involved in structural changes during translation.	✓	P21574
HistoneH4	259	452-710	5'-CGCCAAGCGGCACAGGAAGGTGCTC-3' 5'-GAAGCCGTAGAGAGTGC GGCCCTGGC-3'	11236	Core DNA packaging protein	✓	P02304

Appendix B: Sequenced p52 candidate clones, translation and sequencing primers

XRAP55A: sequenced from IMAGE clone 3405473. Sequence is practically identical to new data bank entry protein with UNIPROT accession no.Q8AVJ2 (IMAGE clone 5571036) Point mutations compared to Q8AVJ2 shown in blue. Translation start shown in green and stop sites shown in red. Numbers refer to nucleotide/amino acid position of the last nucleotide/amino acid of the preceding line.

Shown in orange are the positions of the sequencing primers : 1st- p52F (forward primer), 2nd- p52R (reverse primer).

```

aaggaggacc cgggtttctc gcaatccaca tccatccggg agataccatg agcgggggta 60
ctccatacat cggtagtaag atcagcctga tctccaaggc tgaaatccga tatgaaggca 120
ttttatatac tatcgacacg gaaaactcta cagtagctct cgctaaagtt cgatccttcg 180
gaacagagga cgggccgact gaccgacca tacctccccg agatgaagta tttgaatata 240
ttattttccg tgggaagtgat attaaagacc ttactgtctg tgagccaccc aaacctcagt 300
gttccttgcc tcaggaccct gccattgtac agtcttcatt agggtcctcc tctgcttcc 360
cgttccagtc tgtgagctct tatgggccaat ttggcagaat gcctacatac agtcagttta 420
gcacaagtcc tcttggtggg cagcagtttg gtgctggtgc aggtagttct ttgacctctt 480
ttggagcaga aacaacaagc agcacatctt taccoccaag cagtgttggt ggatccactt 540
tcacgcagga ggcaagaact ttaaaaacac agttatocca aggtcgatca agttcccat 600
tagattcctt aagaaagagc ccaaccattg aacaagctgt gcagactgca tcagcccctc 660
acccccatc ctccgctgct gtaggcagaa gaagccccgt tctgtctaga cccttaccat 720
cctcaagcca aaaaactgct gagagtcccg acaaaggaa aggtgaacta cacaaaatcc 780
agagaccaga cacggagcag aaaaacgatt acaaaaatga cctgagcagg agacaaccag 840
ttttaagtgc tgctcagcct aggagaggtc gagggggaaa tcgaggtggt cgtggaagat 900
ttggagttag aagagatgga cctatgaaat ttgaaaaaga ttctgacttt gaaagtgcta 960
atgcacagtt taacaaagag gacattgaca gagaatttca taataaactg aaattaaag1020
atgataaacc tgagaagcca ttaaaccggag aggataaac cgattctgga gttgacacgc1080
agaacagtga aggccatgca gaggaggagg atgtattggc tgctggagtt tgctactatg1140
ataaaaccaa atctttcttt gatagcatct cctgtgatga taatagggat agaagacagal200
cgtgggcaga agaaagaaga atgaatgctg aaacatttgg cctccctctc cgttcaaaca1260
gaggtcgctg tggttacaga gggagaggtg ggggtatggg cttccgtgga gggagaggcc1320
gtggcggaga gaggagaggt gcaccaggag gtgttggtgg ctttggacct tctcggggtt1380
accgtggtg ttccagagga ggcagaggtg gtagggaatt tgcagaatat gaatacagaal440
aagacaacaa agtagcagcc tagtaaagga tgaaggagtg tccaagtgga ttttataatc1500
atttgacgct cattgaactt gttttcagtc ttgtgaagaa cgaaatttat cttgctgtat1560
attgtcacca gcaactgggg ggggtttatg caaaaaagt gttttagggt ccttctgttt1620
attggctact tctgcacaat ggatccctgt aacactaaag ggtgtagaat gggctttgct1680
cagtctgtat ctattttctt ttttcatttt tgaaaaaaaa aaaaaaatct tta 1733

```

Translation: identical to Q8AVJ2

```
MSGGTPYIGS KISLISKAEI RYEGILYTID TENSTVALAK VRSEFGTEDRP TDRPIPPRDE 60
VFEYIIFRGS DIKDLTVCEP PKPQCCLPQD PAIVQSSLGS SSASSFQSVS SYGPFGRMPT 120
YSQFSTSPLV GQQFGAVAGS SLTSFGAETT SSTSLLPSSV VGSTFTQEAR TLKTQLSQGR 180
SSSPLDSLRLK SPTIEQAVQT ASAPHPPSSA AVGRRSPVLS RPLPSSSQKT AESPEQRKGE 240
LHKIQRPDTE QKNDYKNDLS RRQPVLSAAQ PRRGRGGNRG GRGRFGVRRD GPMKFEKDFD 300
FESANAQFNK EDIDREFHNK LKLKDDKPEK PLNGEDKTDS GVDTONSEGH AEEEDVLAAG 360
VCYYDKTKSF FDSISCDDNR DRRQTWAEER RMNAETFGLP LRSNRGRGGY RGRGGGMGFR 420
GGRGRGGERR GAPGGVGGFG PSRGYRGGSR GGRGGREFAE YEYRKDNKVA A 471
```

P52F:5' CCTTC TCTGCTTCCTTGCCCC 3' (Identical to region of EST with EBI accession number BG408666, starting at position 346):

```
aaggaggacc cgggtttctc gcaatccaca tccatccggg agataccatg agcgggggta 60
ctccatacat cggtagtaag atcagcctga tctccaaggc tgaaatccga tatgaaggca 120
ttttatatac tatcgacacg gaaaactcta cagtagctct cgctaaagtt cgatccttcg 180
gaacagagga ccggccgact gaccgacca tacctccccg agatgaagta tttgaatata 240
ttattttccg tggaagtgat attaaagacc ttactgtctg tgagccaccc aaacctcagt 300
gttccttgcc ttaagaccct gccattgtac agtcttcaat aagggccttc tctgcttctc 360
tgccccaatc tgtgagcttt tatgggccaat ttggcaaaat gcctacatac 410
```

P52R: 3' CC AGCACCAATGTCTCCC 5' (Identical to region of EST with EBI accession number BG486665, starting at position 473 and running backwards)

```
taaagatfff tttttttttt ttcaaaaatg aaaaaagaaa atagatacag actgagcaaa 60
goccattcta cacccttttag tgttacaggg atccattgtg cagaagtagc caataaacag 120
aaggacccta aaacactfff ttggcataaa ccccccccc agtgctggtg acaatataca 180
gcaagataaa tttcgttctt cacaagactg aaaacaagtt caatggactg caaatgatta 240
aaaatccac ttggacaact cttcatcctt tactaggctg ctactttggt gtctttttctg 300
tattcatatt ctgcaaattc cctaccacct ctgcctctc tggaaccacc acggtaaccc 360
cgagaaggtc caaagccacc aacacctcct ggtgcacctc tctctctcc gccacggcct 420
ctccctccac ggaagcccat accccacct ntccctctgt aaccaccag accctctgfff 480
gaacggagag ggaggccaaa tgtttcagca ttcattcttc t 521
```

This published EST runs 3' to 5' of the full transcribed sequence above and is complementary to it. *i.e.* the sequence as read through is the sequence of XRAPA running 3' to 5' and complementary.

XRAP55B: sequenced from IMAGE clone 3473035 and IMAGE clone 4674033. Shown in orange are the positions of the sequencing primers: 1st- RapBsR (reverse), 2nd - RapBsF (forward 1) 3rd - RapBsF2 (forward 2).

```

cgaattgctc agcaacaaa ctgcgggttc tgctctgtcc agcctcctgg tgggtcgggg 60
gggagagagg cggtgccaga ggtgagggtg taaaagggtc agtagtgggc ggccccagg120
cagtctgagg ggatcctgag gagataagtc gcgaggagag agggggccata gtccaaagac180
gagcatcatg agctcaggca ccccgtaacat cggcagcaag atcagtctga tctctaaggc240
tcagatccgc tatgaaggca tctgtacac catcgatacc gagaactcca ctgtggctct300
agccaaagtc cgtcgtttg ggacagaaga tcgtccgact gaccggccag ctccacccc360
ggaggaagtc tacgagtata ttatctccg cggcagcgcac attaaggaca tcaactgtgt420
tgagccccc aaagcgtctc atgctctatc acaggaccct gctatagtcc agtcttctct480
gggctctgca gcttccctacc agccttcagt gccttacagc ccattcagag ggatgccaac540
ctacagccag cttgctgcca ctccctcct gagccaacag tatgctgctt cccttggcct600
cgaaaagttg gggagcccta cggcatctgc cggggcctcc agctcctgct ctcccccctc660
tcctcagcct gtcgccccag aaccagatgt cccggcagag ccaactgcagt tgtctccaaa720
tgctggcttt ccctccatcc cagtgaggaa gagcccaatg gtggaacaag ctgtgcagac780
gggcccactg gagaatcagg cccagaagaa ggttcagcag gcgaaagggg ctctgttgg840
tttgaggggg gttcggcaaa gtggccccc atctcagcca gcacccttaa atgtgccacc900
tccagctgcc ccagttctgg gcacagtaaa tgacgagaac agacgacctc cccggcggag960
atcaggggat agacggacca gaaatcggtc ccgtggacag aacagaccga ctaccgtgaa1020
agaaaacgcc atcaaatttg aaggggactt tgactttgaa tcggccaatg ctcagttcaa1080
cagagaggag ctggataagg agtttaagga taaattaaac ttcaaagatg ataagcctgal1140
aaaggcaggt gaagagaaga ctgattctgg ggtggaaacc cagaatagtg atggaaacccl200
ggaggaagac cctctgggtc caaacacata ttatgaccgg tcaaagtctt tctttgataa1260
tatctcttct gagatgaagt ccagacgcac cacatgggca gaggagagga agctgaatac1320
ggagaccttt ggggtctctg gaaggttctt cagggggcgg agcttccgtg gcggattcag1380
aggaggaaga ggcagtcag ctctctggag gaaccagaca actcagaggg ccggcacagg1440
gcgggtgtga tcagtcactt ctggtacaaa catggacata agggagttat atataattgt1500
tttctctgcc ttcagactga ctgtcttcca tttgtttgt tttctattta cctctttaaa1560
agcattgtag cagttccttg ttaagccaat acccccctc ccccttgctg gtctgatgaa1620
gggcccctgt atgcttcagg tctgcgtttg ggtcttggg acagtgtatg tataggaggt1680
actgagcggg gccacattta cagaggaaag gtcattgtca caagtctgcc gaaaaaggca1740
gaagtttoga acccccacaaa ggaggagtct ctctacctc tttattttaa tgtttgtttt1800
ttccctcct ctccccttg ttattagggg gttttaatgc ttttttttt ttttttttt1860
ttggggtttg tttagcattt tgctcaccct ctatttaatg tacctgttat tttctgcccc1920
tccactttgt gttgctttac aattgagtga caaatgggca cttttagggg tggcggggca1980
tttgattcac agttctcccc atgggcctct aagcatttgt ttaaaagaga ttttaaccgt2040
aataaaaaac cctactcgc cctccgttgc aaatctggca tctgcattta aaggaagcat2100
ctttctctcc gaaaacttct agtcttgacg ggattttcgg tgcaaggcgt gggataattc2160
tggtcccaaa ccctgctat gaccata 2187

```

Translation:

MSSGTPYIGS KISLISKAQI RYEGILYTIID TENSTVALAK VRSEFGTEDRP TDRPAPPREE 60
VVEYIIFRGS DIKDITVCEP PKASHALSQD PAIVQSSLGS AASYQPSVPY SPFRGMPTYS120
QLAATSLLSQ QYAASLGLEK LGSPTASAGA SSSCSSPSPQ PVAPEPDVPA EPLQLSPNAG180
FPSIPVRKSP MVEQAVQTGP LENQAQKKVQ QAKGAPVGLR GVRQSGPQSQ PAPLNVPPPA240
APVLGTVNDE NRRPPRRRSG NRRTRNRSRG QNRPTTVKEN AIKFEGDFDF ESANAQFNRE300
ELDKEFKDKL NFKDDKPEKA GEEKTDSGVE TQNSDGNPEE DPLGPNTYYD RSKSFFDNIS360
SEMKSRRTTW AEERKLNTET FGVSGRFLRG RSFRGGFRGG RGSAAARRNQ TTQRAGTGRV420

COMPOSITIONAL ANALYSIS

A : 34(8.1%); C : 2(0.5%); D : 19(4.5%); E : 29(6.9%); F : 16(3.8%)
G : 34(8.1%); H:-: 1(0.2%); I : 14(3.3%); K : 22(5.2%); L : 22(5.2%)
M : 4(1.0%); N 19(4.5%); P+: 41(9.8%); Q : 23(5.5%); R : 36(8.6%)
S : 46(11.0%); T : 25(6.0%); V : 21(5.0%); W : 1(0.2%); Y : 11(2.6%)

Proline(P) is the most frequently occurring amino acid
Histidine(H) is the least frequently occurring amino acid

CHARGE DISTRIBUTIONAL ANALYSIS

1 0000000000 +00000+000 +0-000000- 0-0000000+ 0+0000--+0 0-+0000+--
61 00-0000+00 -0+-0000-0 0+0000000- 0000000000 0000000000 000+000000
1210000000000 00000000-+ 0000000000 0000000000 0000-0-000 -000000000
181000000++00 00-0000000 0-0000++00 00+000000+ 00+0000000 0000000000
24100000000-- 0++00++00 0++0+0+0+0 00+0000+-0 00+0-0-0-0 -0000000+-
301-0--0++0 00+--0+0+0 0--0+0-000- 0000-000-- -00000000- +0+000-000
3610-0+0++000 0--++000-0 00000+00+0 +00+000+00 +00000++00 000+0000+0

0 : non-charged polar / non-polar amino acid
+: basic amino acid
-: acidic amino acid

RapBsF: 5' TGC TGG CTT TCC CTC CAT CC 3' (Identical to region of EST with EBI accession number BI444610, starting at position 494):

```
tgatctctaa ggctcagatc cgctatgaag gcatcctgta caccatcgat accgagaact 60
cactgtggc totagccaaa gtccgctcgt ttgggacaga agatcgccg actgaccggc 120
cagctccacc ccgggaggaa gtctacgagt atattatattt ccgcggcagc gacattaagg 180
acatcactgt gtgtgagccc ccaaagcgt ctcatgctct atcacaggac cctgctatag 240
tccagtcttc totgggctct gcagcttct accagccttc agtgccttac agcccattca 300
gagggatgcc aacctacagc cagcttgctg cacttcct cctgagccaa cagtatgctg 360
cctcccttgg cctcgaaaag ttggggagcc ctacggcacc tgccggggcc tccagctcct 420
gctcttcccc ttctctcag cctgtcgccc cagaaccaga tgtccggcca gagccactgc 480
agttgtctcc aaatgctggc tttccctcca tcccagtgag gaagagccca atggtggaac 540
aagctgtgca gacgggcca 560
```

RapBsR: 5' CTT CTG TCC CAA ACG AGC GG 3' (Complementary to EST with EBI accession number BI444610 and starting at position 102 running backwards). Used to sequence the nucleotides lying upstream of the sequence in IMAGE clone 3473035 found in IMAGE clone 4674033.

```
tgatctctaa ggctcagatc cgctatgaag gcatcctgta caccatcgat accgagaact 60
cactgtggc totagccaaa gtccgctcgt ttgggacaga agatcgccg actgaccggc 120
cagctccacc ccgggaggaa gtctacgagt atattatattt ccgcggcagc gacattaagg 180
acatcactgt gtgtgagccc ccaaagcgt ctcatgctct atcacaggac cctgctatag 240
tccagtcttc totgggctct gcagcttct accagccttc agtgccttac agcccattca 300
gagggatgcc aacctacagc cagcttgctg cacttcct cctgagccaa cagtatgctg 360
cctcccttgg cctcgaaaag ttggggagcc ctacggcacc tgccggggcc tccagctcct 420
gctcttcccc ttctctcag cctgtcgccc cagaaccaga tgtccggcca gagccactgc 480
agttgtctcc aaatgctggc tttccctcca tcccagtgag gaagagccca atggtggaac 540
aagctgtgca gacgggcca 560
```

RapBsF2: 5' CAG AGG AGG AAG AGG CAG TG 3' (Designed from near complete downstream sequence generated by sequencing with RapBsF primer and identical to sequence starting at position 1378 as shown above).

Overlap of EST BG731378 with BI444610

Overlap is shown in green. Translation start site (not present in IMAGE clone 3743035) highlighted in dark green.

BG731378 (generated from 5' of IMAGE clone 4674033):

```
cgaattgcg agcaacaaag ctgcgggttc tgctctgtcc agcctcctgg tgggtcgggg 60
cgggagaggg cggtgccaga ggtgagggta taaaagggtc agtagtgggc ggcccccagg 120
cagtctgagg ggatcctgag gagataagtc gcgaggagag agggggccata gtccaaagac 180
gagcatcatg agctcaggca ccccgtagat cggcagcaag atcagtctga tctctaaggg 240
tcagatccgc tatgaaggca tctgtacac catcgatacc gagaactcca ctgtggctct 300
agccaaagtc cgctcgtttg ggaccgaaga tcgtccgact gaccggccag ctccaccccg 360
ggaggaagtc tacgagtata ttattttccg cggcagcgac attaaggaca tcaactgtgtg 420
tgagcccca aaagcgtctc atgctctatc acaggaccct gctatagtc agtcttctct 480
gggctctgca gcttctacc agccttcagt gccttacagc ccatte
```

BI444610 (generated from 5' of IMAGE clone 3743035):

```
tgatctotaa ggctcagatc cgctatgaag gcatcctgta caccatcgat accgagaact 60
cactgtggc tctagccaaa gtccgctcgt ttgggacaga agatcgtccg actgaccggc 120
cagctccacc ccgggaggaa gtctacgagt atattatttt ccgcggcagc gacattaagg 180
acatcactgt gtgtgagccc ccaaaagcgt ctcatgctct atcacaggac cctgctatag 240
tccagtcttc tctgggctct gcagcttcc accagccttc agtgccttac agcccattca 300
gagggatgcc aacctacagc cagcttgctg ccacttccct cctgagccaa cagtatgctg 360
cctcccttgg cctcgaaaag ttggggagcc ctacggcacc tgccggggcc tccagctcct 420
gctcttccc ttctcctcag cctgtogccc cagaaccaga tgtcccggca gagccactgc 480
agttgtctcc aaatgctggc tttccctcca tcccagtgag gaagagccca atgggtggaac 540
aagctgtgca gacgggcca
```


XRAPA	KIQRPDTEQKNDYKNDLSRRQPVLSAA - - - - QPRRGRGGNRGGGRGF - G	286
	. : : : : : 	
XRAPB	GVRQSGPQSQPAPLNVPPPAAPVLGTVNDENRRPPRRRSRGNRRTNRNRSG	270
XRAPA	VRR - - - - - DGPMKFEKDFDFESANAQFNKEDIDREFHNKLLKDDKPEKP	331
 : . : : : : . : . . .	
XRAPB	QNRPTTVKENAIKFEGDFDFESANAQFNREELDKEFKDKLNFKDDKPEKA	320
XRAPA	LNGEDKTDSGVDTQNSEGHAEEDVLAAGVCYYDKTKSFFDSISCDNDRD	381
	: : : : : : : . : . : .	
XRAPB	- - GEEKTDSGVETQNSDGN - PEEDPLGPNT - YYDRSKSFFDNIS - SEMKS	365
XRAPA	RRQTWAEERRMNAETFGPLPLRSNRGRGGYRGRGGGMGFRGGRGRGGERRG	431
	. : : . : : : 	
XRAPB	RRTTWAEERKLNTETFGVSGRFLRGR - SFRG - - - - - GFRGGRGSAAPRN	409
XRAPA	APGGVGGFGPSRGYRGGSRGGRRGGREFAEYEYRKDNKVAA	471
	
XRAPB	QTTQRAGTGRV	420

XRAPA / RAP55

Aligned_sequences: 2
1: EMBOSS_001
2: EMBOSS_001
Matrix: EBLOSUM62
Gap_penalty: 10.0
Extend_penalty: 0.5
Length: 478
Identity: 354/478 (74.1%)
Similarity: 393/478 (82.2%)
Gaps: 18/478 (3.8%)
Score: 1804.0

XRAPA	MSGGTPYIGSKISLISKAEIRYEGILY TIDTENSTVALAKVRSFGTEDRP	50
RAP55	MSGGTPYIGSKISLISKAEIRYEGILY TIDTENSTVVLAKFALLGTEDRP	50
XRAPA	TDRPIPPRDEVFEYIIIFRGSDIKDLTVCEPPKPCSLPQDPAIVQSSLGS	100
RAP55	TDRPIPPRDEVFEYIIIFRGSDIKDLTVCEPPKPCSLPQDPAIVQSSLGS	100
XRAPA	SSASSFQSVSSYGPFGRMPTY SQFSTSPLVGQQFG - - - AVAGSSLTSFG	146
	:	
RAP55	SS - TSFQSVSSYGPFGRMPTY SQFNQKSFIRSTKFRGRFRQLNKILRTF -	148
XRAPA	AETTSSTSLPPSSVVGSTFTQEARTLKTQLSQGRSSSPLDSLRSPTIEQ	196
	: : .: :	
RAP55	-ETTSASLSQSSAVGSSFTSDARSLKTQLSQGRSSPHLDTLRKSPTMEQ	197
XRAPA	AVQTASAPHPSSAAVGRRSPVLSRPLPSSSQKTAESPEQRKGE LHKIQR	246
	: 	
RAP55	SVQTPASHLPPPGPVGRRSPPPARPLPSVNQRTIESQEQRVEAHRLSR	247
XRAPA	PDTEQKNDYKNDLSRRQPVL SAAQPRRGRGGNRGGRGRFGVRRDGPMKFE	296
	:.. ..:.. . : : 	
RAP55	PEADQLRNENKDANKRQAAPGAPPARRGRGGHRGGRGRFGI RRDGPMKFE	297
XRAPA	KDFDFESANAQFNKEDIDREFHNK LKLKDDKP - - - EKPLNGEDKTD SGVD	343
RAP55	KDFDFESANAQFTKEEIDREFHNK LKLKDDKPEKVEKPVNGEDKGD SGID	347

XRAPA	TQNSEGHAEEDVLAAGVCYYDKTKSFFDSISCDDNRDRRQTWAEERMN	393
	: : : . : . : : :	
RAP55	TQNSEGNADEEEALASN-CYYDKTKSFFDNISCDDNRERRQTWAEERRIN	396
XRAPA	AETFGLPLRSNRGRGGYRGRGGMGFRGGRGRGGERRGAPGGVGGFGPSR	443
	: : . . : . .	
RAP55	AETFGLPLRSNRGRGGYRGRGSGMGFRGARGRGGQR - - - - -GGFGAPR	439
XRAPA	GYRGGSRGGRGGREFAEYEYRKDNKVAA	471
	: . . : :	
RAP55	GFRGGFRGTRGGREFADFEYRKDNKVAA	467

XRAPA / Q9BX40

Aligned_sequences: 2
1: EMBOSS_001
2: EMBOSS_001
Matrix: EBLOSUM62
Gap_penalty: 10.0
Extend_penalty: 0.5

Length: 486
Identity: 226/486 (46.5%)
Similarity: 263/486 (54.1%)
Gaps: 116/486 (23.9%)
Score: 941.5

```
XRAPA 1 MSG--GTPYIGSKISLISKAEIRYEGILYTIDTENSTVALAKVRSFGTED 48
      ||| |||:|||||:|||||:|||||:|||||
Q9BX40 1 MSGSSGTPYLGSKISLISKAQIRYEGILYTIDTDNSTVALAKVRSFGTED 50

XRAPA 49 RPTDRPIPPRDEVFEYIIFRGSDIKDLTVCEPPKPQCSLPQDPAIVQSSL 98
      |||||.|||:|:|:|||||:|||||.|.:.|||||
Q9BX40 51 RPTDRPAPPREEIYIYIIFRGSDIKDITVCEPPKAQHTLPQDPAIVQSSL 100

XRAPA 99 GSSSASSFQSVSSYGPFGRMPTYSQFSTSPVLVGGQFGAVAGSSLTSFGAE 148
      |:|.|.|. . . .|.|. .|. .|. . . .|.|. :|.|. :|. .|
Q9BX40 101 GSASASPFQPHVPYSPFRGMAPYGPLAASSLLSQQYAASLG----- 141

XRAPA 149 TTSSTSLPSSVVGSTFTQEARTLKTQLSQGRSSSPLDSLRSPTIEQAV 198
                        |..|..|.|. :. |||. :|
Q9BX40 142 -----LGAGFPSIPVG---KSPMVEQAV 161

XRAPA 199 QTASAPH-----PPSSAAVGRRSPLSRPLPSSSQKTAESPEQRKGELH 242
      ||.||. : .|. . . .|. : |. : . . .|. .| |. .
Q9BX40 162 QTGSADNLNAKLLPGKGTGTQ---LNGRQAQPSSKTASD----- 199

XRAPA 243 KIQRPDTEQKNDYKNDLSRRQPVLSSAAQPRRGRGGNRGGRGRF-GVRR-- 289
      :.:|. .|. . . .|. :|| |. :|. .| |. .|. .|. .|. .|
Q9BX40 200 -VVQPAAVQAQGQVNDENRR-----PQRRRSGNRRTNRNRSGQNRPT 240

XRAPA 290 ---DGPMKFEKDFDFESANAQFNKEDIDREFHNKLLKDDKPEKPLNGED 336
      :.:|||. |||||:|:|:|:|. .|. .|. .|. .| | | :
Q9BX40 241 NVKENTIKFEGDFDFESANAQFNREELDKEFKKLLNFKDDKAEK---GEE 287
```

XRAPA	337	KTDSGVDTQNSEGHAEEDVLAAGVCYYDKTKSFFDSISCD-DNRDRRQT	385
		.. . : ... : ... : : . .:.	
Q9BX40	288	K-DLAVVTQSAEAPA-EEDLLGPN-CYDKSKSFFDNISSELKTSSRRTT	334
XRAPA	386	WAEERRMNAETFGLPLRSNRGRGGYRGRGGGMGFRGGRGRGGERRGAPGG	435
		: . :..	
Q9BX40	335	WAEERKLNTETFGVSGRFLRGRS---SRG---GFRGGRGNGTTRRNPTSH	378
XRAPA	436	VGGFGPSRGYRGGSRGGRGGREFAEYEYRKDNKVAA	471
		
Q9BX40	379	RAGTGRV	385

XRAPB	321	-----GEEKTDSGVETQNSDGN-PEEDPLGPNTYYDRSKSFFDNIS-SEM	363
		: . :: : . :.. . :: .:.	
RAP55	333	EKPVNGEDKGDGIDTQNSEGNADDEEEALASNCYDKTKSFFDNIS-CDDN	382
XRAPB	364	KSRRTTWAEERKLNTETFGVSGRFLRGR-SFRG-----GFRGGRGSAAPR	407
		:. . :: . :.. . .: 	
RAP55	383	RERRQTWAEERRINAETFGFLPLRSNRGRGGYRGRGSGMGFRGARGRGG--	430
XRAPB	408	RNQTTQRAGTGRV	420
		
RAP55	431	-----QRGGFGAPRGFRGGFRGTRGGREFADFEYRKDNKVAA	467

XRAPB	TYYDRSKSFFDNISSEMK - -SRRTTWAEERKLNTETFGVSGRFLRGRSFR	394
	. : :	
Q9BX40	CYYDKSKSFFDNISSELKTSSRRTTWAEERKLNTETFGVSGRFLRGRSSR	359
XRAPB	GGFRGGRGSAAPRRNQTTQRAGTGRV	420
	:.. . .	
Q9BX40	GGFRGGRGNGTTRRNPTSHRAGTGRV	385

Appendix D: RAP55/Q9BX40 alignment

Alignments performed with EMBOSS-Align at www.ebi.ac.uk

| indicates identity

- indicates gap imposed by algorithm for optimal alignment

. indicates a mismatch between amino acids

: indicates similarity between paired amino acids: charge or side-chain structure/composition

```
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 488
# Identity:      219/488 (44.9%)
# Similarity:   260/488 (53.3%)
# Gaps:         124/488 (25.4%)
# Score: 867.5
```

```
RAP55    1 MSG--GTPYIGSKISLISKAEIRYEGILY TIDTENSTVVLAKFALLGTED      48
          |||  |||:|||||:|||||:|||||:||||.|||. . . . |||
Q9BX40   1 MSGSSGTPYLGSKISLISKAQIRYEGILY TIDTDNSTVALAKVRSFGTED      50

RAP55    49 RPTDRPIPPRDEVFEYIIFRGSDIKDLTVCEPPKQCSLPQDPAIVQSSL      98
          |||||.|||:|:|||||:|||||.|. : |||||
Q9BX40   51 RPTDRPAPPREEIY EYIIFRGSDIKDITVCEPPKAQHTLPQDPAIVQSSL     100

RAP55    99 GSSSTS-FQSVSSYGPF SRMPTYSQFNQKSFRSTKFGRRFRQLNKILRT     147
          ||:|. | ||. . . |. ||. . . |. . . . . |. . |. : :
Q9BX40  101 GSASASPFQHPVYSPFRGMAPYGPLAASSLLSQQY-----          136

RAP55   148 FETTTASASLSQSSAVGSSFTSDARSLKTQLSQGRSSPHLDTLRKSPTMEQ     197
          :|||  .:|:|. |. |      :..|      |||. : ||
Q9BX40  137 -----AASL----GLGAGFPS-----IPVG-----KSPMVEQ     159

RAP55   198 SVQTT PASH-----LPPPGPV-----GRRSPPPARPLPSVNQRTIESQE     236
          :|||. . |. :      ||. . |. .      ||:|. |. :. . . . |. |
Q9BX40  160 AVQTGSADNLNAKKLLPGKGTGTQLNGRQAQPSSKTASDVVQ-----          202

RAP55   237 QKRVEAHRLSRPEADQLRNENKDANKRQAAPGAPPARRGRGGHRRGGRGRF     286
```

		. . .:... .:.	. .:	
Q9BX40	203	-----PAAVQAQGQVNDENRR-----	PQRR-RSGNRRTRNRS	233
RAP55	287	-GIRR-----DGPMKFEKDFDFESANAQFTKEEIDREFHNKLLKDDKPE		330
		.. :...:	: :	
Q9BX40	234	RGQNRPTNVKENTIKFEGDFDFESANAQFNREELDKEFKKKLNFKDDKAE		283
RAP55	331	KVEKPVNGEDKGDSDGIDTQNSEGNADEEEALASNCYYDKTKSFFDNISCD		380
		: ...: : .. :.. ..	:	
Q9BX40	284	K-----GEEK-DLAVVTQSAEAPA-EEDLLGPNCYYDKSKSFFDNISSE		325
RAP55	381	-DNRERRQTWAEERRINAETFGFLPLRSNRGRGGYRGRGSGMGFRGARGRG		429
	 : . :..	
Q9BX40	326	LKTSSRRTTWAEERKLNTETFGVSGRFLRGRS---SRG---GFRGGRGNG		369
RAP55	430	GQRGGFGAPRGFRGGFRGTRGGREFADFEYRKDNKVAA	467	
			
Q9BX40	370	TTRRN---PTSHRAG-----TGRV	385	

Translation (Q9BX40):

MSGSSGTPYL	GSKISLISKA	QIRYEGILYT	IDTDNSTVAL	AKVRSFGTED	RPTDRPAPPR	60
EEIYEIIFR	GSDIKDITVC	EPPKAQHTLP	QDPAIVQSSL	GSASASPFQP	HVPYSPFRGM	120
APYGPLAASS	LLSQQYAASL	GLGAGFPSIP	VGKSPMVEQA	VQTGSADNLN	AKKLLPGKGT	180
TGTQLNGRQA	QPSSKTASDV	VQPAAVQAQG	QVNDENRRPQ	RRRSNGNRRTR	NRSRGQNRPT	240
NVKENTIKFE	GDFDFESANA	QFNREELDKE	FKKKLNFKDD	KAKEGEEKDL	AVVTQSAEAP	300
AEEDLLGPNC	YYDKSKSFFD	NISSELKTSS	RRTTWAEERK	LNTETFGVSG	RFLRGRSSRG	360
GFRGGRGNGT	TRRNPTSHRA	GTGRV				

References

- Akhtar, B., Wickstrom, J. (1991) Interaction of antisense DNA oligonucleotide analogues with phospholipid membranes (liposomes). *Nucleic Acids Research*, 19; 5551.
- Almouzni G, Wolffe, A.P. (1993) Constraints on transcriptional activator function contribute to transcriptional quiescence during early *Xenopus* embryogenesis. *EMBO Journal*,14; 2033-2047.
- Anderson, J.S.J., Parker, R. (1998) The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. *EMBO Journal*.,17; 1497-1506.
- Andrésson, T., Ruderman, J.V., (1998) The Kinase Eg2 is a component of the *Xenopus* oocyte progesterone-activated signalling pathway. *The EMBO Journal*, 17 (19); 5627-5637.
- Arn, E. A. and Macdonald, P.M. (2001) RNA Localization Goes Direct. *Developmental Cell*, 1; 155-164.
- Bakheet, T., Frevel, M., Williams, B.R., Greer, W., Khabar. K.S. (2001) ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Research*, 29; 246-254.
- Braddock, M., Muckenthaler, M. White, M.R.H., Thorburn, A.M., Sommerville, J., Kingsman, A.J., Kingsman, S.M. (1994) Intron-less RNA injected into the nucleus of *Xenopus* oocytes accesses a regulated translation control pathway. *Nucleic Acids Research*, 22; 5255-5264.
- Bouveret, E., Rigaut, G., Shevchenko, A., Wilm, M., Seraphin, B. (2000) A Sm-like protein complex that participates in mRNA degradation. *EMBO Journal*, 19; 1661-1671.
- Bouvet, P., Matsumoto, K., Wolffe, A.P. (1995) Sequence-specific RNA Recognition by the *Xenopus* Y-Box Proteins. *The Journal of Biological Chemistry*, 270 (47); 28297-28303.
- Castro, A., Peter, M., Magnaghi-Jaulin, L., Vigneron, S., Galas, S., Lorca, T., Labbe, J.C. (2001) Cyclin B/cdc2 induces c-Mos stability by direct phosphorylation in *Xenopus* oocytes. *Molecular Biology of the Cell*, 12; 2660-2671.
- Chang, T.H., Latus, L.J., Liu, Z., Abbott, J.M. (1997) Genetic interactions of conserved regions in the DEAD-box protein Prp28p. *Nucleic Acids Research*, 25; 5033-5040.
- Charlesworth, A., Ridge, J.A., King, L.A., MacNicol, M.C., MacNicol, A.M. (2002) A novel regulatory element determines the timing of Mos mRNA translation during *Xenopus* oocyte maturation. *The EMBO Journal*, 21(11); 2798-2806.

Chen, C.Y., Gatto-Konczak, F., Wu, Z., Karin, M. (1998) Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *Science*, 280; 1945-1949.

Chen, M., Cooper, J.A. (1997) The β subunit of CKII negatively regulates *Xenopus* oocyte maturation. *Proceedings of the National Academy of Sciences USA*, 94; 9136-9140.

Cole, C.N. (2000) mRNA export: the long and winding road. *Nature Cell Biology*, 2; 55-58.

Cullen, B.R. (2003) Nuclear RNA export. *Journal of Cell Science*, 116; 587-597.

Cummings, A., Sommerville, J. (1988) Protein kinase activity associated with stored messenger ribonucleoprotein particles of *Xenopus* oocytes. *Journal of Cell Biology*, 107; 45-56.

Czaplinski K, Ruiz-Echevarria MJ, Paushkin SV (1998) The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. *Genes & Development*, 12(11); 1665-1677.

Darnborough, C.H., Ford, P.J. (1981) Identification in *Xenopus laevis* of a class of oocyte-specific proteins bound to messenger RNA. *European Journal of Biochemistry*, 133; 415-426.

de la Cruz J; Iost I; Kressler D (1997) The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, 94(10); 5201-5206.

de la Cruz, J., Kressler, D., Linder, P. (1999) Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families. *Trends in Biochemical Science*, 24; 192-198.

Deschamps, S., Jacquemin-Sablon, H., Triqueneaux, G., Mulner-Lorillon, O., Potier, M., Le Caer, J., Dautry, F., Le Maire, M. (1997) mRNP3 and mRNP4 are phosphorylatable by casein kinase II in *Xenopus* oocytes, but phosphorylation does not modify RNA-binding activity. *FEBS Letters*, 412; 495-500.

Dumont, J.N. (1972) Oogenesis in *Xenopus laevis*. *Journal of Morphology*. 136; 153-180.

Dupre, A., Jesus, C., Ozon, R., Haccard, O. (2002) Mos is not required for the initiation of meiotic maturation in *Xenopus* oocytes. *EMBO Journal*, 21 (15): 4026-4036.

Evdokimova, V.M., Kovrigina, E.A., Nashchekin, D.V., Davydova, E.K., Hershey, J.W.B., Ovchinnikov, L.P. (1998) Major core mRNP protein p50 promotes initiation of protein biosynthesis *in vitro*. *Journal of Biological Chemistry*, 273; 3574-3581.

- Evdokimova, V.M., Ovchinnikov, L.P. (1999) Translational regulation by Y-box transcription factor: involvement of the major mRNA-associated protein, p50. *International Journal of Biochemistry and Cell Biology*, 31; 139-149.
- Ferrandon, D., Elphick, L., Nusslein-Volhard, C., St.Johnson, D. (1994) Staufen protein associates with the 3'UTR of *bicoid* mRNA to form particles that move in a microtubule-dependent manner. *Cell*, 79; 1221-1232.
- Ferrell, J.E. (1999) *Xenopus* oocyte maturation: new lessons from a good egg. *BioEssays*, 21; 833-842.
- Fischer, N., Weis, K. (2002) The DEAD box protein Dhh1 stimulates the decapping enzyme Dcp1. *EMBO Journal*, 21 (11); 2788-2797.
- Frank-Vaillant, M., Haccard, O., Thibier, C., Ozon, R., Arlot-Bonnemains, Y., Prigent, C., Jesus, C. (2000) Progesterone regulates the accumulation and the activation of Eg2 kinase in *Xenopus* oocytes. *Journal of Cell Science*, 133;1127-1138.
- Gao, M., Wilusz, C.J., Peltz, S.W., Wilusz, J. (2001) A novel mRNA decapping activity in Hela cytoplasmic extracts is regulated by AU-rich elements. *EMBO Journal*, 20; 1134-1143.
- Gebauer, F., Richter, J.D. (1997) Synthesis and function of Mos: the control switch of vertebrate meiosis. *BioEssays*, 19 (1); 23-28.
- Ginisty, H., Sicard, H., Roger, B., Bouvet, P. (1999) Structure and functions of nucleolin. *Journal of Cell Science*, 112; 761-772.
- Gonzalez, C.L., Ruiz-Echevarria, M.U., Vasudevan, S., Henry, M.F., Peltz, S.W. (2000) The yeast hnRNP-like protein Hrp1/Nab4 marks a transcript for nonsense-mediated mRNA decay. *Molecular Cell*, 5;489-499.
- Grosset, C., Chen, C.A., Xu, N., Sonenberg, N., Jacquemin-Sablon, H., Shyu, A. (2000) A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-fos RNA coding determinant via a protein complex. *Cell*, 103; 29-40.
- Gu, W., Tekur, S., Reinbold R., Eppig, J.J., Choi, Y.C., Zheng, J.Z., Murray, M.T., Hecht, N.B. (1998) Mammalian male and female germ cells express a germ cell-specific Y-box protein MSY2. *Biological Reproduction*, 59; 1266-1274.
- Haccard, O., Lewellyn, A., Hartley, R.S., Erikson, E., Maller, J.L (1995) Induction of *Xenopus* oocyte meiotic maturation by MAP kinase. *Developmental Biology*, 168; 677-682.
- Hachet, O., Ephrussi, A. (2001) *Drosophila* Y14 shuttles to the posterior of the oocyte and is required for *oskar* mRNA transport. *Current Biology*, 11; 1666-1674.

Hausen, P., Riebesell, M. (1991) *The Early Development of Xenopus laevis*. Verlag der Zeitschrift für Naturforschung, Tübingen.

Herrick, D., Parker, R., Jacobson, A. (1990) Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. *Molecular Cell Biology*, 10; 2269-2284.

Hilleren, P., McCarthy, T., Rosbash, M., Parker, R., Jensen, T.H. (2001) Quality control of mRNA 3'-end processing is linked to the nuclear exosome. *Nature*, 413; 538-542.

Hock, R., Moorman, A., Fischer, D., Scheer, U. (1993) Absence of Somatic Histone H1 in Oocytes and Preblastula Embryos of *Xenopus laevis*. *Developmental Biology*, 158 (2); 510-522.

Hoffman, D.W., Query, C.C., Golden, B.L., White, S.W., Keene, J.D. (1991) RNA-binding of the A protein component of the U1 small nuclear ribonucleoprotein analyzed by NMR spectroscopy is structurally similar to ribosomal proteins. *Proceedings of the National Academy of Sciences USA*, 88; 2495-2499.

Imtaka, H., Gradi, A., Sonenberg, N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO Journal*, 17; 7480-7489.

Jansen, R-P. (2001) mRNA localization: Message on the move. *Nature Molecular Cell Biology*, 2; 247-256.

Jiang, W., Hou, Y., Inouye, M. (1997) CspA, the major cold-shock protein of *Escherichia coli* is an RNA chaperone. *Journal of Biological Chemistry*, 272; 196-202.

Jones, P.G., Mitta, M., Kim, Y., Jiang, W., Inouye, M. (1996) Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA*, 93; 76-80.

Kajiura-Kobayashi, H., Yoshida, N., Sagata, N., Yamashita, M., Nagahama, Y. (2000) The Mos/MAPK pathway is involved in metaphase II arrest as a cytostatic factor but is neither necessary nor sufficient for initiating oocyte maturation in goldfish. *Development, Genes and Evolution*, 210 (8-9); 416-425.

Keiper, B.D., Rhoads, R.E. (1997) Cap-independent translation initiation in *Xenopus* oocytes. *Nucleic Acids Research*, 25(2); 395-402.

Kistler, A.L., Guthrie, C. (2001) Deletion of MUD2, the yeast homologue of U2AF65, can bypass the requirement for sub2, an essential spliceosomal ATPase. *Genes Development*, 15; 42-49.

Klausner, R.D., Rouault, T.A., Harford, J.B. (1993) Regulating the fate of mRNA: the control of cellular iron metabolism. *Cell*, 72; 19-28.

- Kleene, K. (1996) Patterns of translational regulation during mammalian spermatogenesis. *Molecular Reproduction and Development*, 43; 268-281.
- Kloc, M., Etkin, L.D. (1994) Delocalization of *Vgl* mRNA from the vegetal cortex in *Xenopus* oocytes after destruction of *Xlsirt* RNA. *Science*, 265; 1101-1103.
- Korner, C.G., Wahle, E. (1997) Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. *Journal of Biological Chemistry*, 275; 10448-10456.
- Ladomery, M., Wade, E., Sommerville, J. (1997) Xp54, the *Xenopus* homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Research*, 25 (5); 965-973.
- Le Hir, H., Izaurralde, E., Maquat, L.E., Moore, M.J. (2000) The spliceosome deposits multiple proteins 20-24 nucleotides upstream of exon-exon junctions. *EMBO Journal*, 19 (24); 6860-6869.
- Lejeune, F., Ishigaki, Y., Li, X., Maquat, L.E. (2002) The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodelling. *The EMBO Journal*, 21(13); 3536-3545.
- Libri, D., Graziani, N., Saguez, C., Boulay, J. (2001) Multiple roles for the yeast SUB2/yUAP56 gene in splicing. *Genes Development*, 15; 36-41.
- Lieb, B., Carl, M., Hock, R., Gebauer, D., Scheer, U. (1998) Identification of a Novel mRNA-Associated Protein in Oocytes of *Pleurodeles waltl* and *Xenopus laevis*. *Experimental Cell Research*, 245; 272-281.
- Linder, P., Stutz, F. (2001) mRNA export: Travelling with DEAD box proteins. *Current Biology*, 11; 961-963.
- Luo, M-J., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M., Reed, R. (2001) Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. *Nature*, 413; 644-647.
- Maller, J.L., Butcher F.R., Krebs, E.G. (1979) Early effect of progesterone on levels of cyclic adenosine 3':5'-monophosphate in *Xenopus* oocytes. *Journal of Biological Chemistry*, 254; 579-582.
- Maller, J.L., Krebs, E.G. (1977) Progesterone-stimulated meiotic cell division in *Xenopus* oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase. *Journal of Biological Chemistry*, 252; 1712-1718.
- Manival, X., Ghisolfi-Nieto, L., Joseph, G., Bouvet, P., Erard, M. (2001) RNA-binding strategies common to cold-shock domain- and RNA recognition motif-containing proteins. *Nucleic Acids Research*, 29 (11); 2223-2233.

- Mansfield, J.H., Wilhelm, J.E., Hazelrigg, T. (2002) Ypsilon Schachtel, a *Drosophila* Y-box protein, acts antagonistically to Orb in the Oscar mRNA localization and translation pathway.. *Development*, 129; 197-209.
- Matsumoto, K., Meric, F., Wolffe, A.P. (1996) Translational repression dependent on the interaction of the *Xenopus* Y-box protein FRGY2 with mRNA. Role of the cold shock domain, and selective RNA sequence recognition. *Journal of Biological Chemistry*, 271; 22706-22712.
- Mendell, J.T., Medghalchi, S.M., Lake, R.G., Noensie, E.N., Dietz, H. (2000) Novel upf2p orthologues suggest a functional link between translation initiation and nonsense surveillance complexes. *Molecular Cell Biology*, 20; 8944-8957.
- Mendez, R., Hake, L.E., Andresson, T., Littlepage, L.E., Ruderman, J.V., Richter, J.D. (2000) Phosphorylation of CPE binding factor by Eg2 regulates translation of *c-mos* mRNA. *Nature*, 404 (6775); 302-307.
- Mendez, R., Richter, J.D. (2001) Translational control by CPEB: A means to an end. *Molecular Cell Biology*, 2; 521-529.
- Mitchell, P., Tollervey, D. (2001) mRNA turnover. *Current Opinion in Cell Biology*, 13; 320-325.
- Moss, E., Lee, R.C., Ambros, V. (1997) The cold shock domain protein LIN-28 controls developmental timing in *C.elegans* and is regulated by the *lin-4* RNA. *Cell*, 88; 637-646.
- Murzin, A.G. (1993) OB (oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences. *EMBO Journal*, 12; 861-867.
- Nakamura, A., Amikura, R., Hanyu, K., Kobayashi, S. (2001) Me31B silences translation of oocyte-localising RNAs through the formation of cytoplasmic RNP complex during *Drosophila* oogenesis. *Development*, 128; 3233-3242.
- Navarro, R.E., Yong Shim, E., Kohara, Y., Singson, A., Blackwell, T.K. (2001) *cgh-1*, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C.elegans*. *Development*, 128; 3221-3232.
- Neville, M., Rosbash, M. (1999) THE NES-Crm1p export pathway is not a major mRNA export route in *Saccharomyces cerevisiae*. *EMBO Journal*, 18; 3746-3756.
- Nishizawa, M., Okazaki, K., Furuno, N., Watanabe, N., Sagata, N. (1992) The 'second-codon rule' and autophosphorylation govern the stability and activity of Mos during the meiotic cell cycle in *Xenopus* oocytes. *EMBO Journal*, 11 (7); 2433-2446.
- O'Day, C.L., Dalbadie-McFarland, G., Aberlson, J. (1996) The *Saccharomyces cerevisiae* Prp5 protein has RNA-dependent ATPase activity with specificity for U2 small nuclear RNA. *Journal of Biological Chemistry*, 271; 33261-33267.

- Ohno, M., Segref, A., Kuersten, S., Mattajl, I.W. (2002) Identity elements used in export of mRNAs. *Molecular Cell*, 9; 659-671.
- Palmer, A., Gavin, A.C., Nebreda, A.R. (1998) A link between MAP kinase and p34^{cdc2}/cyclin B during oocyte maturation: p90^{rsk} phosphorylates and inactivates the p34^{cdc2} inhibitory kinase Myt1. *EMBO Journal*, 17; 5037-5047.
- Paynton, B.V. (1998) RNA-binding proteins in mouse oocytes and embryos: expression of genes encoding Y-box, DEAD-box RNA helicases and poly(A)-binding proteins. *Developmental Genetics*, 23; 285-298.
- Pines, J., Rieder, C.L. (2001) Re-staging mitosis: a contemporary view of mitotic progression. *Nature Cell Biology*, 3; 3-6.
- Pitt, J. N., Schisa, J. A. and Priess, J. R. (2000). P granules in the germ cells of *Caenorhabditis elegans* adults are associated with clusters of nuclear pores and contain RNA. *Developmental Biology*, 219; 315-333.
- Posada, J., Sanghera, J., Pelech, S., Aebersold, R., Cooper, J.A. (1991) Tyrosine phosphorylation and activation of homologous protein kinases during oocyte maturation and mitogenic activation of fibroblasts. *Molecular Cell Biology*, 11; 2517-2528.
- Richter, J.D., Smith, L.D. (1984) Reversible inhibition of translation by *Xenopus* oocyte-specific proteins. *Nature*, 309; 378-380.
- Rongo, C., Broihier, H. T., Moore, L., Van Doren, M., Forbes, A. Lehmann, R. (1997) Germ plasm assembly and germ cell migration in *Drosophila*. *Cold Spring Harbour Symposia Quantitative Biology*, 62; 1-11.
- Ross, J. (2001) mRNA turnover. In: *Encyclopaedia of Life Sciences*, Nature Publishing Group, www.els.net.
- Sagata, N. (1997) What does Mos do in oocytes and somatic cells? *BioEssays*, 19 (1); 13-21.
- Schwartz, D.C. and Parker. R. (2000) mRNA decapping in yeast requires dissociation of the cap binding protein, eukaryotic translation initiation factor 4E. *Molecular Cell Biology*, 20; 7933-7942.
- Schwer, B. (2001) A new twist on RNA helicases: DexH/D box proteins as RNPsases. *Nature Structural Biology*, 8(2); 113-116.

- Serin, G., Gersappe, A., Black, J.D., Aronoff, R., Maquat, L.E. (2001) Identification and characterization of human orthologues to *Saccharomyces cerevisiae* upf2 protein and upf3 protein (*Caenorhabditis elegans* SMG-4). *Molecular Cell Biology*, 21; 209-223.
- Sheth, U. and Parker, R. (2003) Decapping and Decay of Messenger RNA Occur in Cytoplasmic Processing Bodies. *Science*, 300; 805-808.
- Smillie, D.A., Sommerville, J. (2002) RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles. *Journal of Cell Science*, 155; 395-407.
- Snay-Hodge, C.A., Colot, H.V, Goldstein, A.L., Cole, C.N. (1998) Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. *EMBO Journal*, 17; 2663-2676.
- Sommerville, J. (1981) Immunolocalization and structural organization of nascent RNP. In: *The Cell Nucleus*, 8, (ed. H. Busch); 1-57. Academic Press, New York.
- Sommerville, J. (1999) Activities of cold shock domain proteins in translation control. *BioEssays*, 21; 319-325.
- Sommerville, J., Lodomery, M. (1996) Transcription and masking of mRNA in germ cells: involvement of Y-box proteins. *Chromosoma*, 104; 469-478.
- Spirin, A.S. (1994) Storage of messenger RNA in eukaryotes: envelopment with protein, translational barrier at 5' side, or conformational masking by 3' side? *Molecular Reproduction and Development*, 38; 107-117.
- Stebbins-Boaz, B., Cao, Q., de Moor, C.H., Mendez, R., Richter, J.D. (1999) Maskin is a CPEB-associated factor that transiently interacts with eIF4E. *Molecular Cell*, 4; 1017-1027.
- Strasser, K. and Hurt, E. (2002) Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. *EMBO Journal*, 19; 410-420.
- Tanner, N.K., Cordin, O., Banroques, J., Doere, M., Linder, P. (2003) The Q Motif. A newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. *Molecular Cell*, 11(1); 127-138.
- Ueno, S., Sagata, N.(2002) Requirement for both EDEN and AUUUA motifs in translational arrest of Mos mRNA upon fertilization of *Xenopus* eggs. *Developmental Biology*, 250; 156-167.

Ullman, K.S. (2002) RNA Export: Searching for mRNA Identity. *Current Biology*, 12; 461-463.

Verlhac, M.-H., Kubiak, J., Clarke, H., Maro, B. (1994) Microtubule and chromatin behaviour follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development*, 120; 1017-1025.

Vilela, C., Velasco, C., Ptushikina, M., McCarthy, J.E. (2000) The eukaryotic mRNA decapping protein Dcp1 interacts physically and functionally with the eIF4F translation initiation complex. *EMBO Journal*, 19; 4372-4382.

Wilusz, C. J., Wormington, M., Peltz, S.W. (2001) The cap-to-tail guide to mRNA turnover. *Nature Reviews*, 2; 237-246.

Yang, J., Bogerd, H.P., Wang, P.J., Page, D.C., Cullen, B.R. (2001) Two closely related human nuclear export factors utilize entirely distinct export pathways. *Molecular Cell*, 8; 397-406.