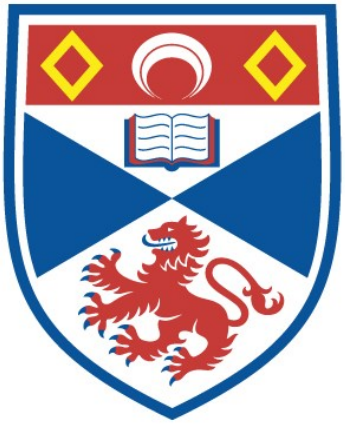
DNA REPLICATION IN THE CHROMOSOMES OF THE CHICKEN, CALLUS DOMESTICUS

Pamela W. McFarlane

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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DNA REPLICATION IN THE CHROMOSOMES OF THE CHICKEN, *Gallus domesticus*

by

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University of St. Andrews

A thesis submitted for the Degree of Doctor of Philosophy

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UNIVERSITY CAREER

I began my University career in the Zoology Department, Bristol University in October 1967 and graduated with an upper second class Honours B.Sc. in June 1970.

In October 1970 I started to work towards a Ph.D. in the Department of Pathology, the Medical School, University of Bristol. There I made some preliminary investigations into the role of the thymus in immunology, with special reference to the functions of a newly discovered protein 'thymolytic factor.'

In April 1971 I married a lecturer at St. Andrews University and applied to continue study towards a Ph.D. in the Zoology Department of the University of St. Andrews. I entered the Zoology Department of the University of St. Andrews in March 1971 and spent three years doing post-graduate research on the pattern of DNA replication in the chromosomes of the chicken Gallus domesticus. The results of the research carried out in the University of St. Andrews are presented here in fulfilment of the requirements for the degree of Doctor of Philosophy.

DECLARATION

I declare that this thesis is the result of my own work. Where observations and experiments performed by others are referred to in the text, they have been acknowledged. Some experiments included in Chapters 1, 2, 3 and 4 have been published in association with H.G. Callan in Journal of Cell Science 13, 821-839 (1973). None of the material in this thesis has been submitted by me for any other degree.

February 25th, 1974.

P.W. McFARLANE
(candidate)

CERTIFICATE

I certify that Mrs. Pamela W. McFarlane has spent 12 terms at research work on DNA replication in the chromosomes of the chicken, *Gallus domesticus*, that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

H.G. CALLAN
(Supervisor)

February, 25th, 1974

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GENERAL INTRODUCTION

DNA synthesis is a process of replication. The complementary nature of the DNA double helix prompted the suggestion by Watson & Crick (1953) that replication is achieved by a semi-conservative mechanism. That DNA replication is indeed semi-conservative was first demonstrated by Taylor *et al.* (1957) and by Meselson & Stahl (1958); both complementary polynucleotide chains of the parent DNA double helix serve as templates for the synthesis of new complementary polynucleotide chains of opposite polarity, and each parent template chain remains in association with the new chain until a further round of replication takes place.

It is now well established not merely that DNA replicates semi-conservatively, but also that the chromatid prior to replication contains only one DNA double helix, i.e. is 'uninsome' (Macgregor & Callan, 1962; Taylor, 1963; Gall, 1963; Miller, 1965; Taylor, 1966; Brewen & Peacock, 1969; Laird, 1971; Kavenoff & Zimm, 1973). These diverse studies contradict the 'multistranded' notion that several or many identical DNA double helices are in parallel, and further they discount the idea that there are protein links between separate lengths of DNA in series within a chromatid prior to replication. Hence the replication of DNA from a single chromatid should reflect the duplication of a single, continuous DNA molecule.

Evidence that the DNA in a eukaryotic chromatid is replicated simultaneously from a number of initiation sites arranged in series along the DNA molecule came initially from the observation of silver grains at multiple sites along a single metaphase chromosome in autoradiographs of cells pulse-labelled with ^{3}H-TdR, (Taylor, 1960, Lima-de-Faria, 1961; Painter, 1961; Stubblefield & Mueller, 1962; Moorhead & Defendi, 1963; German, 1964; Hsu, 1964). Also, it was shown that eukaryotic chromosomes

synthesize DNA more rapidly than could be conceived if there was only one replication unit per chromatid. Such estimates for the rate of DNA synthesis came from experiments in which cells were grown in a medium with a density label, bromouracil, substituted for thymine and the DNA then banded in a CsCl gradient (Painter, et al., 1966; Taylor, 1968). If the DNA in each chromatid were replicated from a single initiation site at the rate calculated by these authors, the time required for the entire chromosome complement of the cell to complete its replication would far exceed the duration of S already established for that cell type.

The study of the replication of chromosomal DNA was transformed by the autoradiographic method devised by Cairns (1962, 1963, 1966), enabling the direct observation in the light microscope of silver grains over extended DNA fibres labelled with tritiated thymidine (^{3}H-TdR). In this technique DNA is isolated using an extremely gentle procedure producing long, unsheared lengths of DNA. On examination of the tracks of silver grains from HeLa cells Cairns discovered that here replication of chromosomal DNA occurs in units arranged in tandem. Since then various adaptations of this technique have been widely used to study DNA replication in other eukaryotes.

Huberman & Riggs (1966, 1968) used the Cairns's technique to investigate DNA replication in Chinese hamster and HeLa cells, and produced the first evidence that each tandem replication unit appears to extend in length by two divergent growing points which arise from a single initiation site i.e. that replication is bidirectional. Lark, et al. (1971) reinvestigated the replication pattern of Chinese hamster cell DNA using a similar techqniue and claimed that replication is not bidirectional but unidirectional. Callan (1972), Amaldi et al. (1972) and Hand & Tamm (1973), however, produced DNA fibre autoradiographs from Triturus and Xenopus.

mouse and Chinese hamster cells, respectively, all in support of a bidirectional model. Later, Huberman & Tsai (1973) reinvestigated their previous findings on Chinese hamster cell DNA and confirmed their original claim. DNA fibre autoradiography was also used by Callan to investigate the relevance of the C-value (the amount of DNA per haploid set of chromatids) and the S-phase duration to the pattern of DNA replication in Xenopus and Triturus. Hand & Tamm and Hori & Lark (1973) studied the effect of puromycin on the rate of DNA replication. Amaldi et al.(1972, 1973) produced evidence which questioned the interpretations of DNA fibre autoradiographs from cells synchronised with 5-fluorodeoxyuridine (FUdR) and investigated the reproducibility of initiation sites from one S-phase to another.

Prokaryotic DNA has been found to be replicated by a single replication unit (Cairns, 1962; 1963) but also bidirectionally (Schnös & Inman, 1970; Bird, et al., 1972; Masters & Broda, 1971; Wake, 1972). DNA synthesis in eukaryotes, unlike that in prokaryotes, must involve coordination of the replication of numerous replication units along a single DNA molecule. This coordination involves the control of specific replication units within each cell, for it is known that in some instances chromosomes, parts of chromosomes or even specific DNA sequences are replicated at defined times during S (Taylor 1960; Hsu, 1964; Lima-de-Faria & Jaworska, 1968; Amaldi, et al., 1969). DNA fibre autoradiography of eukaryotic cells can provide evidence for the rate of DNA replication, the directionality of replication from initiation sites, the distance between neighbouring initiation sites, the correlation in size and time of initiation of neighbouring replication units, and whether there are different populations of distinct replication units.

The aim of the present work was to study the replication units of

the chicken genome and to enquire what such units might reflect. For example:-

(1) C-value. The size of replication units might be expected to be proportional to the amount of DNA present within the cell. If the unineme model of chromosome organization holds, then the DNA molecule in a chromosome from an organism with a high C-value is expected to be longer than that from an organism with a low C-value. Organisms with a high C-value may have long replication units if the increased DNA represents a greater number of repetitive sequences and if these repeated copies of a single gene or group of genes are replicated together as a single unit.

The chicken has a very low C-value, 1.45 pg (computed from Atkin, et al., 1965) and therefore might be expected to show very short replication units, shorter than, for example, replication units from mammalian tissues, which have a C-value of about 3 to 3.5 pg (see review by Callan, 1972).

(2) S-phase. The S-phase duration may change considerably during development. This means that DNA must be replicated at different rates in different cells of the same organism. It is usually the case that the S-phase of embryonic cells is much shorter than S in somatic cells and therefore DNA replication must be more rapid in the former. The increase in rate might be effected by a faster rate of travel of replication forks along the DNA molecule, or by an increase in the number of coincidentally active initiation sites arranged in tandem. Callan (1972) found the latter to be true in Triturus neurulae, which have an S-phase of about 4 to 6 hours compared to an S-phase of some 9 to 10 days at pre-meiosis. A question to be answered is whether this is a general rule for DNA replication in eukaryotes. For this reason DNA fibre autoradiographs were prepared from chick blastoderms

and compared with those from chick somatic cells; the S-phases for both cell types were also determined.

(3) Cell type. As cells differentiate different genes are transcribed within different cells. A question arises as to whether there is any relation between the size of transcription and replication units. If there is, then the replication units in the relatively undifferentiated chick blastoderm might be different from the replication units measured from DNA fibre autoradiographs of chick somatic cells.

(4) Type of DNA. Is the nucleitide sequence of the parent polynucleotide chain reflected in the size or the time of initiation of the replication units? Satellite or highly repetitive DNA occupies about 5% of the total DNA from whole chick cell nuclei and is located mainly in the heterochromatic fraction (Comings & Mattoccia, 1972a). The chicken also has unusually small microchromosomes (Plate 3) which differ from the macrochromosomes or large chromosomes in that their DNA shows a non-Gaussian skewing to the heavy side of the main band of DNA when analysed by analytical ultracentrifugation; this is known as heavy shoulder DNA (Comings & Mattoccia, 1972b). Satellite, heavy shoulder, or other 'types' of DNA may be characterized by having a different replication pattern from the rest of the DNA. For this reason it would be of interest to investigate whether distinct and different populations of replication units occur in DNA fibre autoradiographs from chick somatic cells.

GENERAL ACCOUNT OF MATERIALS AND METHODS

(a) TISSUE CULTURE

(i) Preparation of materials

All glassware was first rinsed and old labels removed; it was then soaked for several hours in a dilute solution of the detergent, RBS 25. After washing, the glassware was rinsed with 6 changes of tap water, or continuously for one hour in tap water, then rinsed twice in deionised water.

(ii) Sterilisation

Glassware, wrapped in foil, was sterilized by dry heat at 145°C for 2 hr. Screw caps, magnetic stirrers etc. were autoclaved at 15 lb pressure for 20 min. Small amounts of tissue culture solutions were filtered using Millipore discs, HA (0.45 µm) fitted with a Swinney adaptor to a 20 ml disposable syringe. Dissecting instruments were sterilised by boiling for 30 min in deionized water. Before use, the air in the tissue culture room was sterilized overnight by ultra-violet light. During experiments the working surfaces beneath a sterile hood were frequently swabbed with 70% alcohol.

(iii) Aseptic technique

To prevent contamination of sterile materials, from the air, the tissue, and myself, standard precautions and manipulations e.g. flaming of bottle tops, were adhered to during the setting up of a cell culture.

(iv) Sterility testing

If contamination of a solution or a cell culture was suspected, this was tested by inoculation of previously prepared Nutrient Agar (Oxoid) plates. Several drops of the suspected solution were smeared across the plates. After incubation at 37°C for several days most fungal and bacterial colonies would have been detected. Suspect contamination was checked by microscopical examination.

(v) Solutions

Medium 199 - with Hanks's salts (Morgan, et al., 1950) was supplied by Flow Laboratories, Ltd. Cultures set up in Petri dishes and incubated in a 5% CO_2/95% air-circulated incubator, were grown in medium 199 containing 35 g/l sodium bicarbonate. Cultures grown in equilibrium with air, in closed screw-capped flasks, were set up in medium 199 without sodium bicarbonate.

Foetal Calf Serum (FCS) was supplied by Flow Laboratories, Ltd. and stored at - 20°C.

Penicillin/Streptomycin (penstrep) 5000 i.u. of each per ml, was supplied by Flow Laboratories, Ltd. and stored at - 20°C. (The term growth medium designates medium 199 + 8% FCS + 50 i.u./ml penstrep).

Tyrode's balanced salt solution (BSS), Tyrode (1910), was made up according to the method outlined by Paul (1970), aliquoted into 100 ml bottles and stored at 4°C.

Tyrode's balanced salt solution lacking Ca^{++} and Mg^{++} salts (Moscona, 1952) was made up according to the method outlined by Paul (1970). This

adaptation of the standard salt solution was made to avoid the natural tendency of the cells to stick to one another and form clumps when washed.

0.25% trypsin (Difco 1:250) was made up in Tyrode's BSS lacking Ca^{++} and Mg^{++} salts. This concentration gave tissue disruption without significant cell damage over 90 min.

0.5% trypsin (Difco 1:250) was diluted in citrate buffer (Paul, 1970). Subculturing of overgrown cell monolayers was carried out by suspending cells from the monolayer with 0.5% trypsin; the trypsin was then removed, the cells resuspended in fresh medium, and replated at a lower concentration.

(vi) Culture procedure for somatic cells

Fertile eggs supplied by Edinburgh Poultry Research Centre were incubated in a water-circulated incubator at 37°C for 9 to 11 days. Before opening, the egg shells were wiped with 70% alcohol. The shell was cracked at the air space with blunt forceps, and the shell membrane peeled away. The embryo was then lifted out with curved forceps and placed in BSS, previously warmed to 37°C, in a covered Petri dish. Two or three embryos were used to establish a single primary culture. The heads and limbs were removed and the trunks washed and transferred to a second dish of BSS at 37°C. The trunks were chopped in pieces with sprung scissors for about 10 min until a soup-like consistency was obtained. This material was transferred to centrifuge tubes and spun down at or below 1000 rpm (higher speeds result in cell damage). The supernatant was discarded, the pellet washed twice with BSS and once with BSS lacking Ca^{++} and Mg^{++} salts, to remove red blood cells. The pellet was subsequently resuspended in 30 ml of 0.25% trypsin in BSS lacking Ca^{++} and Mg^{++} in a flask containing a small

magnetic stirrer. This material was enzymatically disrupted with gentle stirring at 37°C. After 30 min the material was allowed to settle, and the supernatant discarded. A further 30 ml of the 0.25% trypsin saline was added and the process repeated. After 30 min the material was allowed to settle and the supernatant collected. This was repeated and again the supernatant collected. The two supernatants were then combined, spun at 1000 rpm for 3 min, and the resulting pellet of cells resuspended in 10 ml of growth medium. Cell concentration was assessed with a haemocytometer, and the tissue culture flasks seeded at a concentration 3 to 5 x 10^5 cells/ml. The correct concentration for seeding was assessed empirically from the time taken for formation of a monolayer of the ideal cell concentration. The cells were incubated at 37°C until the log growth phase was reached. The growth medium was changed when necessary, usually about every 2 days. The monolayer was generally subcultured every 4 days. A primary culture of somatic cells became unhealthy after some 10 days; it was then discarded, and a fresh primary culture established. Plate 1 is a photomicrograph of a chick somatic cell monolayer taken under phase contrast. Plate 3 shows a diploid chick cell at metaphase.

(vii) Isolation of blastoderms

Fertile chicken eggs were incubated at 37°C for periods ranging from 11½ to 18 hr. After incubation, each egg was wiped with 70% alcohol and the shell broken in half. The contents of the egg were collected in a covered Petri dish and the thin albumen removed by transferring the egg from one Petri dish to another. The yolk was then positioned so that the blastoderm was uppermost. The yolk membrane was gently lifted and grasped with fine forceps at a point about 1 cm from the blastoderm. The blastoderm was removed by carefully cutting the yolk membrane at about 2 mm distance

from the blastoderm and the blastoderm plus membrane lifted out into a small Petri dish containing BSS previously warmed to 37°C. Discrete masses of yolk granules adhering to the underlying yolk membrane were removed with forceps without disrupting the blastoderm. Cleaned blastoderms were then placed in a second dish of BSS lacking Ca^{++} and Mg^{++} at 37°C and gently disrupted by pipetting. Attempts to remove the blastoderm from its underlying yolk membrane proved too difficult to be adopted. The material was then centrifuged at or below 1000 rpm for 3 min and the pellet of cells, membrane and adhering yolk granules resuspended in growth medium previously warmed to 37°C.

Plate 2 shows an intact blastoderm after 18 hr incubation. This stage in development represents Stage 3+ (Hamburger & Hamilton, 1951), the intermediate streak stage. The primitive streak is relatively long and broad. No primitive groove is present.

(b) CELL CYCLE ANALYSES

(i) S-phase in somatic cells

A primary culture of chick somatic cells was established, and the experiment was begun when the cells appeared to have reached log growth, 24 to 48 hr after plating. The old culture medium was poured away and fresh pre-warmed medium containing radioactive thymidine (^{3}H-TdR) was added. A pulse of low activity ^{3}H-TdR (2 Ci/mMol: final concentration 3 μCi/ml) was applied for 15 min to cell monolayers in twenty-two 25 cm^2 flasks. After labelling, the culture medium was removed and fresh pre-warmed medium containing non-radioactive thymidine (TdR) at 500 times the molarity of the previously applied ^{3}H-TdR, was added. At hourly intervals

over 22 hr a flask was removed for processing, time 0 being taken as the termination of the labelling period. The cell monolayer was trypsinized and the cells harvested. After a slight hypotonic treatment (2 to 10 min in 20% BSS) the cells were fixed in 3 parts absolute alcohol : 1 part glacial acetic acid directly on slides. This technique involved the resuspension of the cells in about 0.1 ml of hypotonic solution (20% BSS); a small drop of cells was then taken up in a narrow bore pipette and a drop of fixative in a second pipette. The fixative was pipetted onto a clean slide and the cells dropped onto the spreading fixative. After about 20 sec the drop of cells began to flatten. At this time a second drop of fixative was placed on to the spreading drop and the hypotonic solution driven away and wiped off. An easily distinguishable circle of cells, with well-spread metaphases, resulted.

Preparations were filmed with NTB 2, exposed for 6 to 10 days, developed, stained with Giemsa and examined. The percentage of labelled to total mitoses was scored (see Plate 4), at least 500 mitoses being examined for each point recorded.

(ii) Cell generation time in somatic cells

A primary culture of chicken fibroblasts was established; some thirty 25 cm^2 tissue culture flasks were set up at 3 x 10^5 cells/ml. The cells reached log growth about 48 hr after plating and were used at this stage. The culture medium was poured away and fresh pre-warmed medium containing ^{3}H-TdR, 2 µCi/ml (specific activity 2 Ci/m Mol) was added.

At 2-hourly intervals over 22 hr two flasks were removed for processing. Time 0 was taken as the time at which label was added.

The cell preparations were prepared according to the method outlined on p. 11. These slides were then filmed with NTB 2, exposed for 7 days, developed, stained with Giemsa and examined. The percentage of labelled cells to total cells was scored, no fewer than 500 cells being examined for each point recorded. From this experiment an estimate for the mean length of $G_1 + G_2 + M$ was established. T is found by adding to this value the value previously found for S. T, G_1, G_2, M, and S is the standard nomenclature for stages in the cell cycle (Howard & Pelc, 1953).

(iii) S-phase in the blastoderm

A rough estimation for the S-phase duration in the blastoderm was determined by scoring the percentage of labelled cells in a random cell population after a pulse-label of ^{3}H-TdR for 15 min. This gives a mean estimate for the fraction of time in the cell cycle occupied by S. The mean duration of $G_1 + G_2 + M$ was then found by continuously labelling for about 15 hr with ^{3}H-TdR a population of cells derived from these blastoderms, and subsequently scoring the labelled fraction of cells in the cell population as a function of time as above. The duration of S was then calculated from the equation outlined below. (This estimation assumes that the total cell number remains constant. The blastoderm, like cells in tissue culture is, however, most likely to be an exponentially growing population of cells. No information is available from this study whereby corrections can be made to the calculations to allow for exponential growth (Cleaver, 1967). For this reason this estimation for the duration of S must only be considered a rough approximation).

x = labelled fraction after pulse labelling.
y = the duration of $G_1 + G_2 + M$.

(a) If x% of T = S

then $\frac{x}{100} \times T = S$ $\quad\therefore T = \frac{100\,S}{x}$

(b) $G_1 + G_2 + M + S = T$ $\quad\therefore S = T - y$

By substitution from (a) $\quad S = \frac{100\,S}{x} - y$

or $S = \frac{x\,y}{100 - x}$

Determination of labelled cell fraction

Three fertile chicken eggs were incubated at 37°C for 18 hr. After incubation the blastoderms were isolated and immediately placed in 3 separate small Petri dishes each with 4 ml of growth medium containing ^{3}H-TdR (concentration 4 µCi/ml, Specific activity 2 Ci/m Mol) pre-warmed to 37°C. Isolated blastoderms were kept submerged below the surface of the medium in the Petri dish because cells may lyse at the liquid/air interphase. Embryos at this early stage in development are extremely fragile, and for this reason the isolated blastoderms varied in the degree to which they remained intact. Some broke down to form dispersed cells in the medium, while other retained the shape of the blastocyst in vivo.

Care was taken not to allow the temperature of the isolated embryos to fall below 37°C and Petri dishes containing the isolated blastoderms were quickly returned to the incubator. These cells were allowed to continue growth in the presence of ^{3}H-TdR for 15 min.

Labelling was terminated by pouring the contents of each Petri dish into 3 separate, pre-cooled test tubes and plunging these tubes into crushed ice. The tubes were centrifuged at 1000 rpm for 3 min and the pellet of cells washed in hypotonic solution (20% BSS). Slides of such cells were prepared according to the method outlined on p. 11 . These were then filmed with NTB 2, exposed for 6 to 8 days, developed, stained with Giemsa and examined.

The percentage of labelled cells to total cells was estimated in a total of 500 cells.

Determination of the duration of $G_1 + G_2 + M$

32 fertile chicken eggs were incubated for $11\frac{1}{2}$ to $12\frac{1}{2}$ hr at 37°C. Blastoderms were isolated in two batches, 22 after $11\frac{1}{2}$ hr incubation and 10 after $12\frac{1}{2}$ hr incubation. Each blastoderm was isolated directly into growth medium pre-warmed to 37°C and containing ^{3}H-TdR (concentration 4 µCi/ml, Specific Activity 2 Ci/m Mol). Each blastoderm was placed in a separate Petri dish and quickly returned to the incubator at 37°C. Every hour over 16 hr two Petri dishes were removed and labelling terminated by plunging the cells to 0°C. Duplicates were taken each time because it was difficult to determine at a cursory inspection whether blastoderms were viable or not.

Slides were prepared as on p. 11 and examined. The percentage of labelled to total cells as a function of time was scored for cells labelled for 1 to 16 hr. Plate 5 shows a whole cell autoradiograph prepared from a chick blastoderm, continuously labelled for 12 hr.

(c) CELL SYNCHRONY

For certain experiments semi-synchronously growing somatic cells were obtained by the addition to the medium of 5-fluorodeoxyuridine (FUdR), an inhibitor of thymidine biosynthesis, at 4×10^{-6} M. This block can be overcome by supplying thymidine from an exogeneous source. FUdR mimics thymidine as it is phosphorylated in most cells to fluorodeoxyuridylate, which inhibits thymidylate synthetase, thus blocking the conversion of decxyuridylate to thymidylate (Cohen, et al. 1958). As this is the sole pathway for thymidylate synthesis, and the thymidylate pool is usually very small (Taylor, et al., 1962) DNA replication is quickly blocked. Fluoro-uridylate, produced by conversion of FUdR, is known to be incorporated into

RNA (Gordon & Staehelin, 1958); an excess of uridine (0.5 µg/ml) was therefore added to prevent RNA synthesis from being halted.

Somatic cells were treated with FUdR and uridine for 16 hr, a period somewhat longer than the mean duration of $G_1 + G_2 + M$ (Fig. 2). Those cells that are in S when the FUdR is applied will be blocked in S and the majority of cells in G_1, G_2 and M when the FUdR is first applied should be synchronized and blocked at the beginning of S.

The effective cell synchrony i.e. the number of cells blocked at the beginning of S, was measured by comparing the percentage of cells found in S in a normal asynchronous population of cells with that percentage found in S from a population of cells treated with FUdR and uridine. A primary culture of chick somatic cells was established. When the cells had reached log growth, about 48 hr after plating, fresh pre-warmed medium was applied to 3 control flasks and fresh pre-warmed medium containing FUdR and uridine applied to 3 experimental flasks. After 16 hr, the medium from both the experimental and control flasks was removed and replaced with radioactive medium (growth medium + ^{3}H-TdR, 2 Ci/m Mol, at a final concentration of 4 µCi/ml) for 15 min. The cells were then trypsinized and harvested. After a short hypotonic treatment (20% BSS for 2 to 10 min) they were fixed in 3 parts absolute alcohol : 1 part glacial acetic acid directly on slides according to the method outlined on p. 11 . Preparations were filmed with NTB 2, exposed for 6 days, developed, stained with Giemsa, and examined. The percentage of labelled cells, i.e. cells in S, to total cells, was calculated for both control and experimental cultures, about 1200 cells being counted in each case. The effect of pre-treatment of chick somatic cells with FUdR and uridine for 16 hr is shown in Plate 6.

In certain experiments chick blastoderms incubated for 18 hr were also treated with FUdR before labelling. In such experiments, however, FUdR was not applied in order to synchronize the cells to any great extent, but rather to block the formation of thymidylate and so reduce the pool of thymidylate in these cells.

Blastoderms were isolated directly into growth medium containing FUdR (1 µg/ml, 4 x 10^{-6} M), and uridine (0.5 µg/ml), pre-warmed to 37°C The embryos, each in separate Petri dishes, were quickly returned to the incubator. Incubation was continued for 2 hr. The FUdR-medium was then centrifuged off, (1000 rpm for 3 min) and the cells placed in labelled medium.

(d) LABELLING PROCEDURES

DNA fibre autoradiographs were made from cells labelled with high specific activity ^{3}H-TdR (23 to 28 Ci/mMol) at a final concentration of 50 µCi/ml, for periods between 15 and 120 min. In other experiments, referred to as pulse-chase experiments, two labelling regimes were employed consecutively. Some experiments involved a standard pulse-chase procedure. After a determined period of labelling in high specific activity ^{3}H-TdR, the labelled medium was removed and replaced with non-radioactive medium, in which the cells continued to grow for a further determined period. During the chase period a progressive dilution of ^{3}H-TdR occurs as labelled molecules are withdrawn from the pool, and one expects this to be reflected in a progressive reduction of the radioactivity of the DNA synthesized. If, however, the cells' thymidylate pool is very small a progressive reduction in the radioactivity of DNA may not be detected. Thus in some experiments a deliberate dilution of the radioactivity was made at the beginning of the chase period by adding to the labelled medium 3 times the quantity

of cold thymidine as the ^{3}H-TdR previously present. Another schedule involved a pulse of label followed by a step-up in the specific activity of the label rather than a reduction. The specific activity of the ^{3}H-TdR was initially reduced to ¼ of its activity by the addition of non-radioactive thymidine. This medium containing low activity label was supplied for a determined period, then poured away, and labelling continued with medium containing high specific activity ^{3}H-TdR for a further determined period.

The method of labelling somatic cells was as follows: medium from asynchronous cell cultures, or from cells growing in medium plus FUdR and uridine, was removed and the cells washed in BSS at 37°C. Radioactive medium was added, and incubation continued until at the pre-determined time the pulse label was either terminated and the cells harvested, or the chase or specific activity change provided and the cells subsequently harvested. After trypinization the cells were washed in BSS lacking Ca^{++} and Mg^{++}, both the trypsin and salt solution containing FUdR and uridine. Termination of labelling or chase was carried out in earlier experiments by quickly resuspending the washed cells in concentrated sucrose solution. In later experiments cells were kept at 0°C immediately following trypsinization, because it was assumed that this procedure would terminate DNA synthesis more positively.

Blastoderms (18 hr incubation) were labelled according to the same procedures. The blastoderms were dissected from the yolk and placed either directly into growth medium containing ^{3}H-TdR or first into growth medium + FUdR and uridine for 2 hr and then into the labelled medium. After the labelling or chase period the cells were plunged to 0°C and the labelled medium centrifuged off. These cells were then washed in BSS, centrifuged, and resuspended in sucrose solution ready for dialysis.

(e) PRODUCTION OF DNA FIBRES

The technique of DNA fibre autoradiography was first devised by Cairns (1962, 1963, 1966) and later developed by Huberman & Riggs (1968). The method used in the present study follows that of Huberman & Riggs with a few subsequent modifications introduced by Callan (1972).

At the termination of labelling, the cells were trypsinized, harvested, and in certain experiments cooled to 0°C. Serial dilutions of the concentrated cell suspensions were made in a strong sucrose solution (1 M sucrose, 0.05M NaCl, 0.01M EDTA, pH adjusted to 8 with NaOH). The suspensions were mixed well, and 1 ml aliquots were transferred to dialysis chambers. Each dialysis chamber was made by fusing a ring, about 25 mm o.d., of Pyrex rod, about 4 mm o.d., to Pyrex tubing, o.d. also about 4 mm; parallel flats were then ground on the ring. Millipore filters (V.M. 50 µm) 25 mm in diameter were stuck to the chambers with Durofix adhesive, the shiny surface of the filter to the inside of the chamber. The chambers were left to dry out for several days before use. Aliquots of cell suspensions were transferred to such dialysis chambers using a long, thin siliconized pipette, filling from the bottom up so as to exclude air bubbles.

Precautions concerning the thorough mixing of the cell suspensions were taken to ensure an even distribution of fibres on the filters when finally drained. Serial dilutions of the cells were performed, because it was difficult to pre-determine the ideal cell concentration per chamber needed to produce a sparse but sufficient distribution of fibres on a filter. After several experiments it was concluded that an initial concentration of about 10^5 cells per chamber and about four serial dilutions produced DNA fibre autoradiographs of the desired density. All further treatments were carried out at 25°C in a constant temperature bath. The filled dialysis chambers were stood upright in cord-bored stands over glass

containers into which the various dialysis solutions were poured. The cell suspensions were first dialysed against a lysis medium (1% sodium dodecyl sulphate, SDS, in sucrose/NaCl/EDTA, pH adjusted to 8 with NaOH) for from 4 to 6 hrs. The function of the viscous sucrose solution both inside and outside the dialysis chamber was to minimise breakage of the DNA fibres when the cells were lysed. The chambers were then dialysed against 0.05 M NaCl, 0.01 M EDTA, pH adjusted to 8 with NaOH, for about 1 hr, to remove the SDS and sucrose, and subsequently dialysed overnight against 0.1% pronase in standard citrate saline, SSC-tris (0.15M NaCl, 0.015 M Na_3 citrate, 0.01 M tris, pH 8). The pronase and the digested cell proteins were then washed away from the DNA in the chambers by dialysis against 4 changes of dialysis medium, (0.05 M NaCl, 0.01 M EDTA pH 8), each of 2 hr. Each chamber was lifted from the dialysis medium, held upright, and any solution trapped above an air-bubble, which might not have been subjected to dialysis, was removed with a finely-drawn pipette. The bottom edge of one or both filters was then pierced with a needle, and the contents allowed to drain out slowly. The concentration of the DNA within a single chamber was judged by its stickiness, and both drag and blocking during draining was avoided by drawing out the contents with filter paper. As the chambers are drained some of the bare DNA fibres stick to the filters at certain points, and are pulled down along the line of drainage. The chambers and filters were allowed to dry out overnight and the filters then detached from the rim of the chambers with a razor blade. The glued edge of each filter was then cut away and the filters exposed to concentrated formalin vapour in covered chambers for 2 hrs. This step in the procedure was introduced by Huberman & Riggs to denature any residual pronase, which might digest the autoradiographic film which was later to be applied.

Each filter was then attached to the middle of a clean 3" x 1" microscopy slide with 4 pin points of Durofix adhesive, so as to flatten the filter. These slides had been previously cleaned by immersion in chromic acid overnight; they were then rinsed in boiling water and finally placed in 75% alcohol before being dried by Kimwipe paper. The slides carrying filters were allowed to dry thoroughly so as to discharge any vapours coming from the adhesive, and were then covered with Kodak AR 10 stripping film. These preparations were exposed for 28 to 34 weeks.

(f) AUTORADIOGRAPHY

(i) DNA fibre autoradiography

Kodak AR 10 stripping film was applied to the preparations according to the standard procedure. The stripping film was cut on the plate into rectangles of approximately 65 by 25 mm, stripped off with forceps and placed sensitive side down on a clean surface of distilled water. The temperature of the materials and the room was kept at 20°C. The piece of film was then draped over the filter by moving the slide below the piece of film on the water surface, lifting the film out and tilting the slide to right and left. The slides were then placed on a rack to dry thoroughly before being packed away in light-tight boxes and stored at 4°C.

The processing of AR 10 autoradiographs over Millipore filters involved certain modifications from the usual procedure. The slides were developed in total darkness for 20 to 30 min in D 19 at 20°C. The long development time was employed to create large silver grains on the film. The slides were rinsed twice in distilled water (because the filters tend to trap D 19 which would precipitate with hypo), and then fixed for about 5 min in Metafix. Each slide was then placed, back down, in an

individual dish containing water, and the preparation washed gently in running tap water for about 10 min before being rinsed in distilled water. Such a slide was then placed in a deeper dish of distilled water, and the back of the film cut through. Both the film and the extreme edge of the filter were grasped firmly with two pairs of forceps and the film gently pulled away from the underlying filter. The filter was discarded, and the slide meantime cleaned. The clean slide was then passed below the film such that the delineated margin of the filter on the film was well positioned, and the film once more draped over the slide by tilting to right and left. The preparations were drained and dried and subsequently immersed in 2% formalin for about 10 min to harden the film on the slide. The slides were finally washed again in running tap water, this time for over 1 hr, rinsed in distilled water and dried in racks over a hot plate. The film from the back of the slide was scraped away before the film was thoroughly dried.

This elaborate washing procedure was introduced because it is not possible to wash the detached film thoroughly without causing excessive swelling of the film. Nevertheless the fixative must be entirely removed if the silver grains are not to fade.

(ii) Autoradiography of whole cell preparations

Labelled preparations of cells prepared for cell cycle and cell synchrony analyses were filmed with NTB 2 dipping emulsion according to the usual procedure. NTB 2-coated slides were exposed for from 6 to 8 days, and were developed for 2½ min in D 19.

(g) CELL AND CHROMOSOME STAINS

Whole cell autoradiographs, filmed with NTB 2, developed and air dried, were stained with 4% Giemsa (G.T. Gurr's stain) made up to 50 ml in 0.01 M phosphate buffer, pH 6.5 to 6.8 for 1 hour. To avoid the scum formation inherent in all Giemsa staining techniques Giemsa was added to the slides which had previously been placed in a Coplin jar containing the buffer. Slight warming while staining increased the intensity of the stain. Stained preparations were then rinsed in distilled water and air dried. Slides were examined uncovered.

Whole cell preparations from blastoderms were prepared according to the method outlined on p. 11 and were stained according to various procedures, Ehrlich's haematoxylin and eosin, Weigert's haematoxylin, and in 4% Giemsa, pH 6.5. In all cases both cells and yolk granules were stained. The distinction between yolk granules (which varied in size from 4 to 20 μm) and cells proved to be too difficult in cell preparations stained with either haematoxylin stain. Giemsa staining was found to be the most useful, for yolk granules tended to be pink whereas cells tended to the purple-blue, the cell nuclei a deeper blue.

Air-dried chromosome preparations were made according to the method outlined on p. 11 and stained overnight in 2% Aceto-orcein (G.T. Gurr, synthetic) freshly filtered before use. After a brief rinse in 45% Acetic acid, the preparations were rinsed in 95% alcohol, two changes of absolute alcohol, each of 3 min duration, and two changes of xylene; they were subsequently mounted in Canada balsam (see Plate 3).

(h) MICROSCOPY AND PHOTOGRAPHY

DNA fibre autoradiographs were examined by bright field microscopy using an oil-immersion lens (Zeiss planapochromat x 40); immersion oil was subsequently removed by rinsing in petroleum ether and acetone. Xylene must not be used because it causes the silver grains to fade.

Lengths of tracks, and distances between replication origins, were measured using a x 12.5 KPL focussing eyepiece containing a scale, x 40 objective, and Optovar set to 1.25. This set up was checked to give 50 ocular divisions for 100 µm.

Photographs of DNA fibre autoradiographs were taken using Pan F photographic film with light and filters adjusted for exposure times of about 4 sec.

Unstained chromosome preparations were examined using phase contrast microscopy. Stained chromosome preparations and stained whole cell autoradiographs were examined using bright field microscopy. Photographs of metaphase cells were taken on Pan F film using an Ilford 807 filter plus a Zeiss broad band interference filter, oil immersion lens (Neofluar x 100), Optovar x 1.6, x 8 KPL eyepiece and at an exposure time of 3 sec.

Autoradiographs of stained whole cells were photographed using Pan F film with bright field microscopy.

CHAPTER I.

CELL CYCLE ANALYSES

(a) S-phase duration in chick somatic cells

Introduction

The period of DNA synthesis in the cell cycle (T) is called the S-phase. S is preceded by a period, G_1, and succeeded by a period G_2 which is followed by mitosis (M) and the end of the cycle. This nomenclature was introduced by Howard & Pelc (1953). The technique used here to determine the S-phase was one of the earliest to be employed in studying the cell cycle (Howard & Pelc, 1953) and was first outlined in its complete form by Quastler & Sherman (1959).

Method

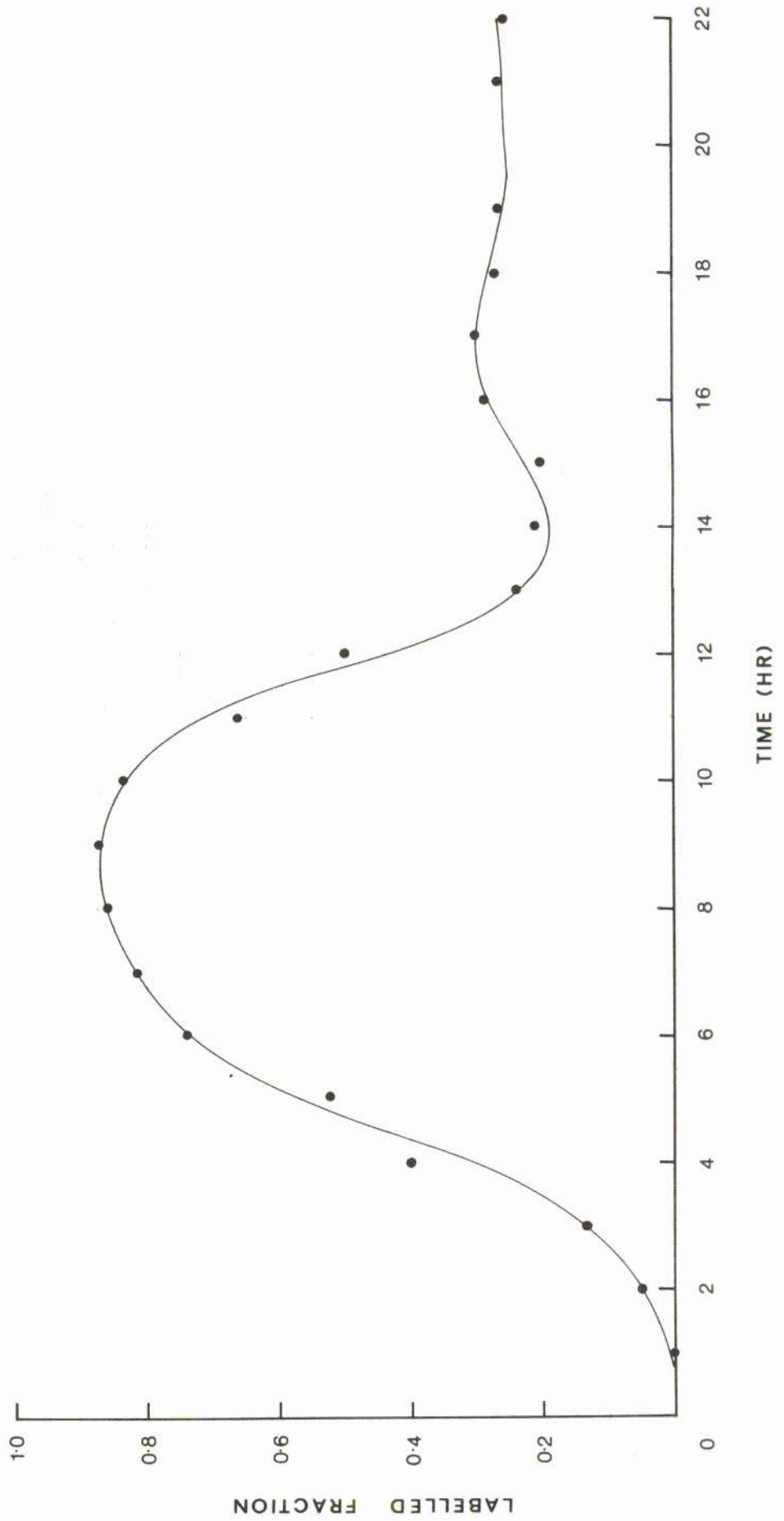
The method for culturing cells and determining S is outlined in the General Account of Materials and Methods. Chick somatic cells at 37°C are pulse labelled for 10 min with ^{3}H-TdR and then chased with pre-warmed medium containing TdR. Two S-phase determinations were performed on two separate primary cell cultures. Each point in Fig. 1 is derived from the average value from both experiments.

Results

Plate 4 is a photograph of an autoradiographic preparation prepared from chick somatic cells collected 8 hr after the pulse label of ^{3}H-TdR was added. Labelled and unlabelled mitoses and interphase cells can be observed. Fig. 1 shows the fraction of labelled mitoses in preparations taken from cultures fixed at hourly intervals from 0 to 22 hr after the

Fig. 1. The fraction of labelled mitoses in chick somatic cells in culture as a function of time following a short pulse of ^{3}H-TdR.

Fig 1


LABELLED FRACTION
1·0
0·8
0·6
0·4
0·2
0
2
4
6
8
10
12
14
16
18
20
22
TIME (HR)

pulse label was added. The earliest samples have no labelled mitoses. The proportion of labelled mitoses then rises rapidly as cells in S when the ^{3}H-TdR was applied, and therefore labelled, go through G_2 and into mitosis. Following the peak there is a trough as unlabelled cells, originally in G_1 when the ^{3}H-TdR was added, enter mitosis. The duration of the S period is taken as the time between the two points on the curve where 50 per cent of the mitoses are labelled. The time from the mid-point of the labelling pulse to that at which 50% of the mitoses are first labelled is equal to $G_2 + \frac{1}{2}$ M. Half the mitotic time is added to G_2 because the mitoses are scored only in metaphase, which means that the cells have to go through not only G_2 but also prophase before they are scored; prophase lasts for roughly half the time of mitosis in most cells (Mitchison, 1971). The 0.5 points (50%) give the times at which half of the cell population has reached certain stages of growth and for this reason the phase durations obtained will be the median rather than the mean for the population. If some cells in the population are dead or are progressing extremely slowly through the cell cycle, the labelled fraction will not reach 1.0. In Fig. 1 the plateau is reached at about 0.86, i.e. 14% of the total cell population is not viable, or is not dividing normally. The 0.5 point is therefore actually taken at 0.43. The median value for S-phase duration is therefore about 7.5 hr and the value for $G_2 + \frac{1}{2}$ M, about 4.5 hr.

Discussion

Previous values for the S-phase duration in chick somatic cells have been shorter, 5 to 6 hr (Cameron, 1964; Fujita, 1962; Baserer, 1968, & Liebecq-Hutter, 1965). It has been assumed from these results that birds in general have shorter S-phases than mammalian cells (Cleaver, 1967; Mitchison, 1971). Two of these estimates are from *in vivo* studies

(Cameron, 1964; Fujita, 1962) and therefore cannot be compared to the *in vitro* estimate obtained in the present study. As Cameron has suggested, the shorter S-phase of bird cells compared to mammalian cells observed in his *in vivo* studies may be related to the higher body temperature of birds (41°C) compared to that which is maintained in mammals (37°C). The two remaining *in vitro* studies at 37°C were carried out using diverse techniques. Liebecq-Hutter (1965) calculated the S-phase duration in a population of cells synchronized by mitotic selection. Basleer (1968) used Feulgen microspectrophotometry to find the fraction of the population in S in an unsynchrhonized population and derived a value of the S-phase duration from a value for T calculated by Chèvremont *et al.* (1961). Basleer's value must be considered very approximate because no attention is paid to the percentage of viable cells and the diversity that must occur between different cell populations. However, the main reason for the discrepancy between the above estimates and that derived in the present study is, in my opinion, because variations in the value of S occur between different cell cultures of the same tissue, perhaps especially in primary cultures. These differences may arise from variations in culture conditions or from variations in the cells themselves. Differences in S-phase duration are observed even in established cell lines. For example, Cleaver (1967) and Fujiwara (1967) found the duration of S in Mouse L cells to be between 10.5 and 13.5 hr whereas several other authors, including Killander & Zetterberg (1965) and Stanners & Till (1960), find S in these cells to be 6 to 8 hr.

In vitro estimates of G_2 in chick somatic cells also vary. Firket & Verly (1958) estimate G_2 to be about 7 hr while Chèvremont *et al.* (1961) estimate G_2 to be some 3 hr. My estimate for the duration of $G_2 + \frac{1}{2}$ M, 4.5 hr, lies between these values.

If S is 7.5 hr in chick somatic cells *in vitro* at 37°C then this value is similar to the average *in vitro* estimate for the S-phase duration in mammalian cells, 6 to 8 hr (See review by Cleaver, 1967). This comparison is important if the replication pattern of the DNA in chick somatic cells determined by DNA fibre autoradiography is to be compared with DNA fibre autoradiographic studies on mammalian cells.

(ii) Duration of T in chick somatic cells

Introduction

The cell cycle time (T) is the time taken for a cell to progress completely around the cell cycle. This figure is the same as the population doubling time if only viable cells are considered. The population doubling time is usually calculated by measuring the increase in the number of cells in a population over a long period. This method was found unsatisfactory in the present study because the plating efficiency of these cells was below 50%. The cell cycle time was thus determined by calculating the duration of $G_1 + G_2 + M$ by a continuous labelling technique and adding to this figure the value obtained for S-phase duration determined above. Both an S-phase determination and the estimate of $G_1 + G_2 + M$ was determined on the same primary culture of cells.

Method

The methods used are outlined in the General Account of Materials and Methods.

Results

Fig 2 shows the fraction of cells that are labelled in the total cell population as a function of time in the presence of continuously applied ^{3}H-TdR. This fraction increases steadily with the duration of labelling due to the steady entry of cells into S. The fraction of cells labelled initially represents those cells which were in S at the start of labelling. The proportion of labelled cells increases steadily as cells in G_1, G_2 and M at the start of labelling, enter S.

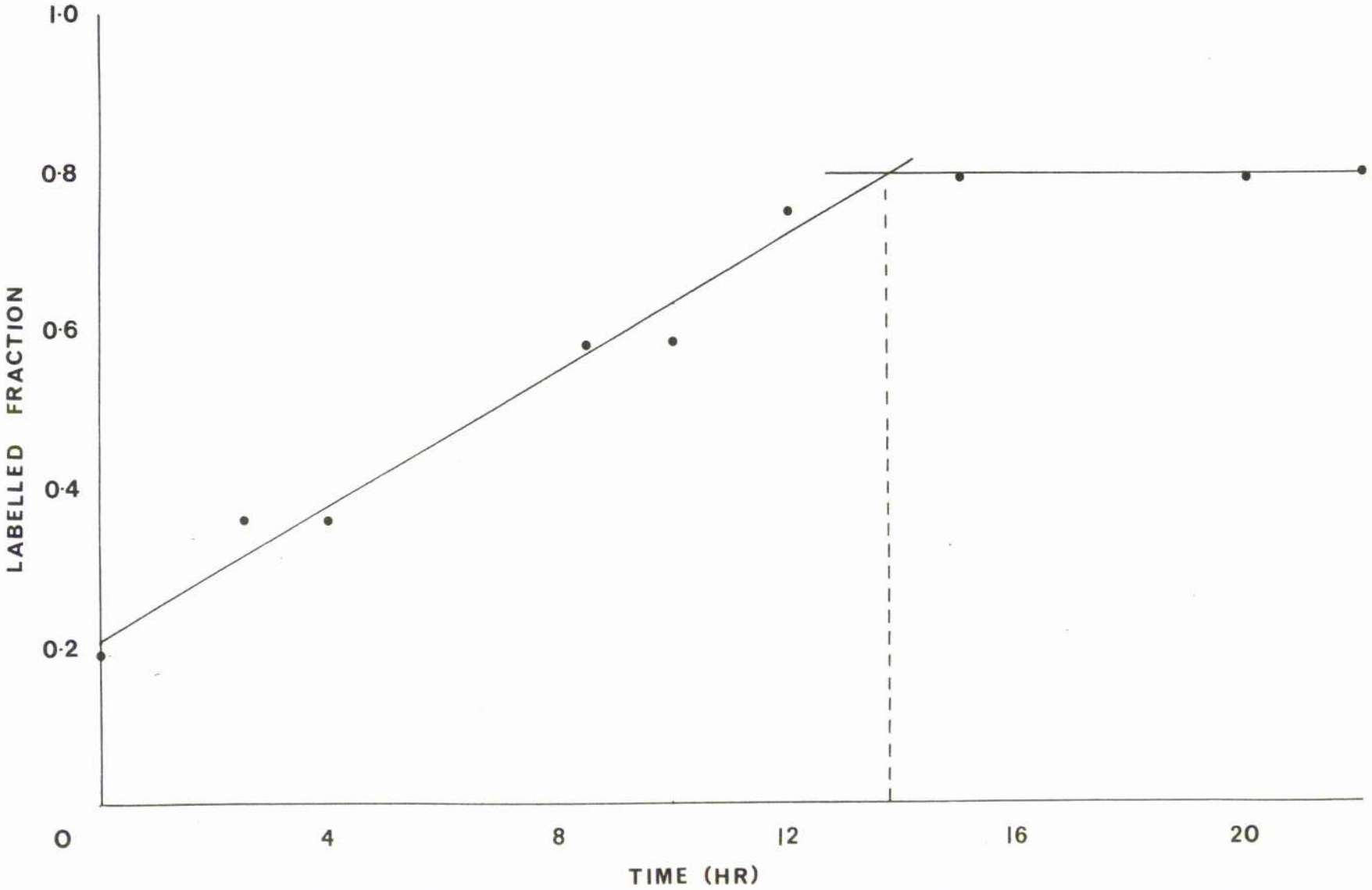
If all the cells in the population are viable or are progressing through the cell cycle at or near the normal rate, the labelled fraction should reach 1.0 after a period of time equal to $G_1 + G_2 + M$. The labelled fraction does not reach 1.0, however, but flattens out at about 0.8. This means that 20% of the cell population represents dead cells or cells progressing very slowly through the cell cycle. The maximum level for the fraction of cells labelled is taken to be 0.8 because it remains at this level for some 7 hr. The 'best-fitting' line for the slope in Fig. 2 can be defined as the line for which the sum of the squares of deviations of the predicted y-values from the observed y- values is a minimum. (The observed values are the dots, the predicted values are on the line and the deviations would be vertical lines that connect the observed and predicted values). The slope of the line and the y- intercept were found according to a standard equation.

$$b = \frac{\text{Sum of } xy - \frac{(\text{Sum of } x)(\text{Sum of } y)}{n}}{\text{Sum of } x^2 - \frac{(\text{Sum of } x)^2}{n}}$$

b is the slope of the line, n equals the number of points, x and y the observed values of the dots on the graph. The y-intercept is $(\bar{y} - b.\ \bar{x})$ where $\bar{y} = \frac{\text{Sum of } y\ i}{n}$ and $\bar{x} = \frac{\text{Sum of } x\ i}{n}$

The slope and the plateau cross at a point equal to 13.75 hr.

Fig. 2. The fraction of labelled cells in a chick somatic cell culture as a function of time in the presence of continuously applied ^{3}H-TdR.


1·0
0·8
0·6
0·4
0·2
0
LABELLED FRACTION
4
8
12
16
20
TIME (HR)

This time represents the mean duration of $G_1 + G_2 + M$ in the cell population. The value for T is obtained by adding to this figure the previously estimated value for S-phase duration, 7.5 hr. The average cell cycle time of chick somatic cells is therefore estimated to be 21.25 hr under the specified growth conditions. The value for G_1 may also be obtained by subtraction. If $G_1 + G_2 + M = 13.75$ hr and $G_2 + \frac{1}{2} M = 4.5$ hr (Fig. 1) then $G_1 + \frac{1}{2} M = 9.25$ hr. If mitosis is assumed to be about 1 hr (Review by Cleaver, 1967) then $G_1 = 8.75$ hr.

Discussion

Previous *in vitro* estimates for the length of T in chick somatic cells at 37°C have been shorter, 15 hr (Lièbecq-Hutter, 1965), 13 hr (Chèvremont, *et al.*, 1961). As maintained on p. 26, variations in the phases of the cell cycle in different experiments probably stem from variations in culture conditions, in the cells themselves and in the degree of accuracy of the techniques used to obtain such values.

The duration of the cell cycle in chick somatic cells, obtained in the present study, is similar to the duration of the cell cycle *in vitro* obtained for many mammalian cells at the same temperature, 37°C. For example, various human cell types in culture have generation times of between 16.8 and 42 hr, though in most human cell types T is some 18 to 28 hr. (See review by Cleaver, 1967).

A value for the duration of the cell cycle is required in order to synchronize cultures of chick somatic cells with FUdR, according to the method outlined on p. 15. FUdR blocks cells at the G_1/S interphase. To obtain the maximum number of cells blocked at the G_1/S interphase an unsynchronised population of cells is treated with FUdR for a period of time somewhat longer than $G_1 + G_2 + M$.

(iii) S-phase duration in the chick blastoderm

Introduction

The S-phase duration in the chick blastoderm was not determined by the method on p. 10 , (the scoring of the fraction of labelled mitoses as a function of time after a pulse label) because the identification of mitoses proved to be difficult in these cells. Mitoses were not well spread, were weakly stained and were very often obscured by overlying yolk granules. Mitotic cells could of course be distinguished, but only by careful observation and staining. The mitotic index in blastoderms incubated for 18 to 21 hr was 3%. This was found by incubating eggs for 18 to 21 hours, dissecting out the blastoderms and making cell preparations according to the method on p. 11. Thirty mitoses were noted in 1000 cells examined. This percentage is thought to be near the expected value for this stage blastoderm (Stage 3+, Hamburger & Hamilton, 1951), if one extrapolates from the data presented by Emanuelsson (1965).

The S-phase duration was determined by finding the percentage of cells labelled in the total population following a brief pulse of ^{3}H-TdR. This value is, to a first approximation, proportional to the fraction of time in T that is occupied by S. The duration of $G_1 + G_2 + M$ was then found by plotting the increase in the labelled fraction of cells as a function of time, in the presence of continuous label as in the method on p. 12 for somatic cells. The mean duration of S. was then calculated from the equation shown on p. 12.

Both the above values were determined from blastoderms isolated in vitro, for two reasons. First, labelling experiments for DNA fibre autoradiographs of blastoderms were more conveniently carried out in vitro

(Chapter 5) and the duration of S in cells isolated in the same manner was therefore required. Secondly, to achieve continuous labelling of blastoderm cells *in vivo* it is necessary to give repeated injections or continuous infusion of label and this may well result in disturbances at the cellular level, which make cell cycle analyses difficult to interpret.

In certain experiments outlined in Chapter 5, DNA fibre autoradiographs were prepared for blastoderms incubated for 18 hr *in vivo*, then isolated *in vitro* and treated with FUdR for 2 hr after which they were labelled for up to 60 min. For this reason the duration of S in blastoderms between 18 and 21 hr old was required. The continuous labelling method for estimating $G_1 + G_2 + M$, takes several hours, however, i.e. it exceeds the expected duration of S. This determination was therefore carried out on blastoderms incubated *in vivo* and *in vitro* for $11\frac{1}{2}$ to 27 hr. The estimate finally obtained was assumed to be a rough approximation for the mean duration of S in a 18 to 21 hr old blastoderm.

Method

The methods for determining (i) the labelled fraction after pulse labelling and (ii) the duration of $G_1 + G_2 + M$ are outlined in the General Account of Materials and Methods.

Results

(i) Labelled fraction

1000 cells were examined from pulse-labelled chick blastoderms of which 452 were labelled. The labelled fraction in the total population was therefore 0.45.

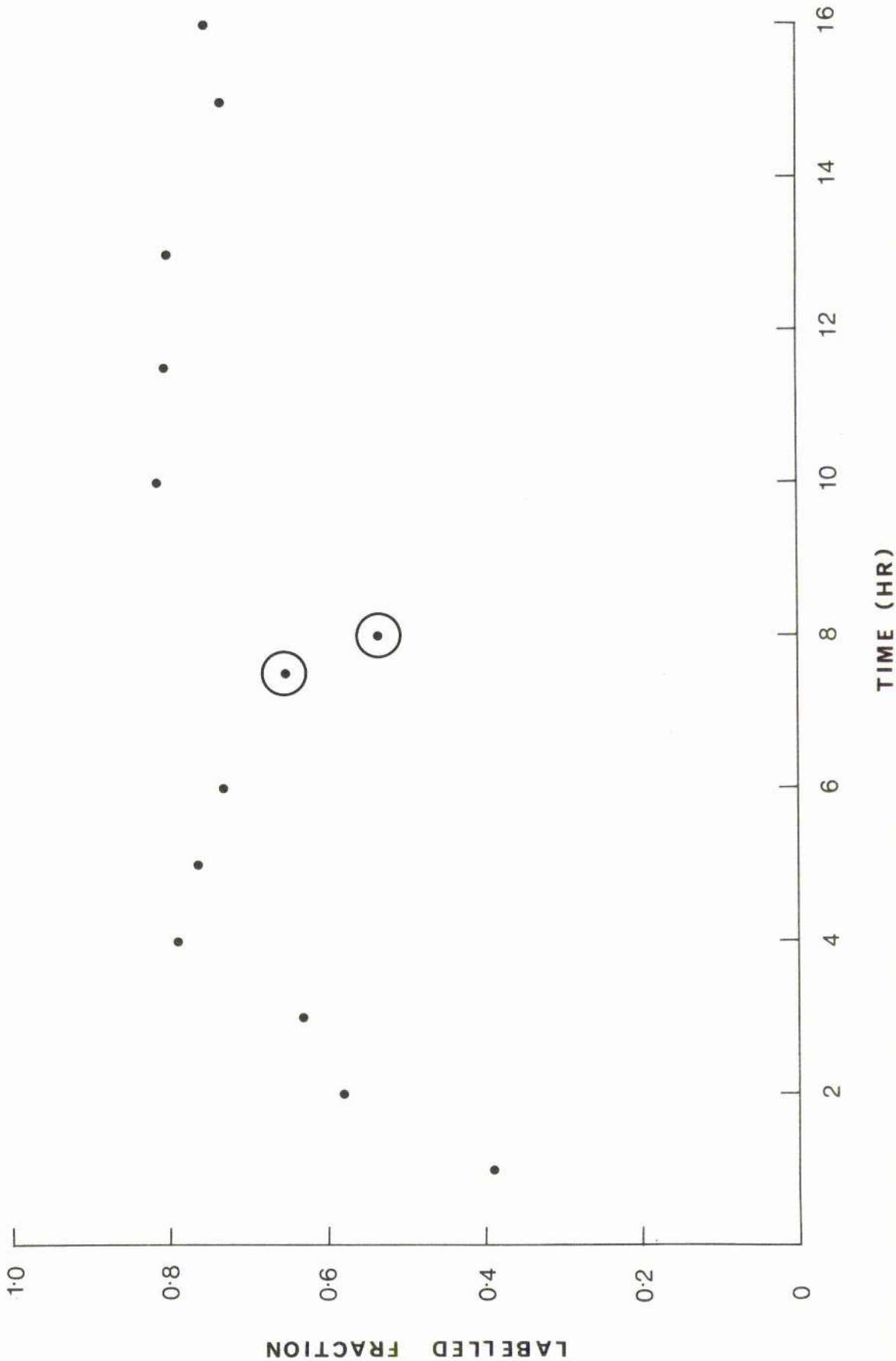
(ii) Duration of $G_1 + G_2 + M$

Fig. 3 shows the fraction of labelled/total cells as a function of time during continuous labelling over 16 hr. Two experiments were run concurrently i.e. two blastoderms in separate Petri dishes were labelled for each time period, 2 blastoderms were labelled for 1 hr, 2 blastoderms were labelled for 2 hr, etc. Slides were made from both experiments in each case. 500 cells from slides from both experiments were examined for each point recorded on the graph. No significant variation was noted in the labelled fraction from slides prepared from either experiment after each labelling period. Plate 5 shows a whole cell autoradiograph of chick blastoderm cells harvested after 12 hours in medium containing ^{3}H-TdR.

A steady rise in the labelled fraction is expected in Fig. 3 until a plateau is reached when all cells are labelled. The time taken for the labelled fraction to reach its maximum is proportional to the mean duration of $G_1 + G_2 + M$. A steady rise is not observed, however. The labelled fraction after 1 hr labelling is 0.4. It then rises sharply and steadily over 4 hr to 0.78. This labelled fraction is not maintained over the remainder of the labelling period as expected, but falls to 0.54 after $7\frac{1}{2}$ hours in the presence of ^{3}H-TdR. This is followed by an abrupt rise to 0.81 after 10 hr labelling. A plateau is then maintained over several hours until the experiment was terminated, 16 hours after the beginning of labelling. Ignoring, for the moment, the fall in the labelled fraction between about $7\frac{1}{2}$ and $9\frac{1}{2}$ hr labelling, the initial rise in the labelled fraction to a plateau at 0.78 occurred 4 hours after the beginning of labelling, giving an estimate of 4 hr for $G_1 + G_2 + M$. On the other hand, the graph may be interpreted in such a way that $G_1 + G_2 + M$ equals 10 hr, if the second maximum is taken.

Fig. 3. The fraction of labelled cells in 11½/12½ hr incubated chick blastoderms isolated *in vitro* as a function of time in the presence of continuously applied ^{3}H-TdR. There is reason to suspect the validity of the 2 points enclosed in circles.

Fig 3



Discussion

The labelled fraction of the total cell population after pulse labelling chick blastoderm cells in vitro was 0.45. This fraction should be similar to that obtained after 1 hr in the continuous labelling experiment, Fig. 3, i.e. 0.39. This difference demonstrates the variability that may occur between experiments.

In Fig. 3 the labelled percentage does not rise above 81%. This means that about 20% of the cells in the population are dead or are stationary in G_1 or G_2 or are progressing exceedingly slowly round the cell cycle. The labelled fraction is therefore not 0.45 but 45/81 cells, 0.56. Because the number of dead or 'slow' cells will most likely increase as incubation of the blastoderm in vitro continues and cells become unhealthy, 0.56 is probably still an underestimate.

The labelled fraction first reaches near maximum (0.78) after 4 hours labelling. I would consider this to be the correct estimate for the mean duration of $G_1 + G_2 + M$ for the following reason. A fall in the labelled fraction in a continuously labelled population can be explained in only one way: the blastoderms labelled for $7\frac{1}{2}$ and 8 hours must have been unhealthy due to circumstances such as culture medium variations, temperature irregularities etc. If these two readings are thus explained and discounted, a reasonably steady plateau would result.

If $G_1 + G_2 + M$ equals 4 hr and the fraction of the cell cycle occupied by S is approximately 0.56, the duration of S must equal about 5 hours, as determined from the equation on p.12 .

The duration of the cell cycle and its integral phases are often shorter in embryonic or young tissues than in the adult. (Reviewed by

Mitchison, 1971). Mammals, however, may be a notable exception as T has been found to be very similar in both early cleavage stages and most adult somatic tissues in the mouse, some 10 to 20 hr (Graham, 1971); there is, however, no information for the length of S in the early cleavage stage.

The value of S in the chick blastoderm is slightly shorter than the value for S determined for somatic cells, 7.5 hr. It must be remembered, however, that these figures were calculated using different techniques. If the shorter S-phase duration in the chick blastoderm is valid, the difference is by no means as marked as it is in the embryos of some invertebrates and Amphibia (see review by Cleaver, 1967). Even though S is not much shorter in the chick blastoderm as compared to S in the adult, about 60% of the cell cycle is here occupied by S, whereas in somatic cells in culture S represents only about 20% of the cycle (From Figs. 1 and 2).

$G_1 + G_2 + M$ lasts 4 hr in the chick blastoderm, considerably less than in chick somatic cells, 13.75 hr. G_1 and G_2 may be reduced because cell growth is not of prime importance at and following cleavage, as is thought to be the case in embryonic tissues of other organisms (Mitchison, 1971).

To the best of my knowledge there have been no previous estimates for the S-phase duration in the early chick embryo. Emanuelsson (1965) found that the duration of T increases steadily with age and is 7.4 ± 2.0 hr in chick blastoderms *in vivo* in eggs secured immediately at the moment of laying. According to the present study the duration of T is 10 hr in chick blastoderms incubated over $11\frac{1}{2}$ to 27 hr *in vitro* and *in vivo*. An increase in the duration of T is expected as the time of incubation is greater in the blastoderms used in the present work. If one assumes the

younger the embryo the shorter the time spend in G_1 and G_2, (and this seems quite likely from the present study), S would probably be only slightly less than the value for T, 7.4 ± 2.0 hr, in the unincubated blastoderm. If the duration of S increases steadily with T as incubation takes place, a value of 5 hr for S in a 1½ to 27 hr-incubated blastoderm is within the range expected.

CHAPTER 2.

PULSE-CHASED CELLS

INTRODUCTION

Early in 1966 Cairns, working on HeLa cells, obtained the first DNA fibre autoradiographs from eukaryotic cells. Later in 1966 Huberman & Riggs produced similar DNA fibre autoradiographs from Chinese hamster cells. Huberman & Riggs prepared DNA fibre autoradiographs from cells labelled with ^{3}H-TdR for 35 to 40 hr and demonstrated labelled DNA fibres up to 1.8 mm in length. Fundamental questions concerning the validity of the technique were posed and answered in this paper. It was confirmed that the silver grain tracks on the autoradiographs were indeed produced by the incorporation of ^{3}H-TdR into chromosomal DNA. Huberman & Riggs also demonstrated that these silver grain tracks, and the labelled lengths of single extended DNA fibres that produced them, were the same length. Cairns's DNA fibre autoradiographs not only demonstrated labelled sections of DNA over 500 µm in length but from cells labelled for 45 and 180 min, produced the first experimental evidence that the long labelled DNA fibres were composed of many separately replicated, tandemly joined sections. In 1968, Huberman & Riggs, using a pulse-chase labelling procedure found, unexpectedly, that replication proceeded in opposite directions from each initiation site.

The present experiments represent a continuation of the above studies of the replication of eukaryotic DNA. DNA fibre autoradiographs of chick somatic cells in culture at 37°C were prepared from cells pre-treated with FUdR and labelled according to the pulse-chase procedure. The pulse-chase protocol was employed to provide evidence for the direction of DNA replication, the timing or sequence of initiations, the distance between initiation sites, the relationship between adjacent initiation intervals and the presence

or absence of defined termini. FUdR pre-treatment was carried out in order to semi-synchronise the cell population.

Any track of silver grains on an autoradiograph is referred to as a section or track throughout this study. The term replication unit refers to a track of silver grains that is thought to represent DNA replicated by the progression of two diverging growing forks that arise from a common origin. A growing fork is defined as any one site on a parental double stranded DNA molecule where replication has resulted in the production of two daughter strands of DNA. An initiation site or origin is defined as that region in a replication unit where DNA synthesis is initiated. The term initiation interval refers to the distance between two adajcent initiation sites.

METHOD

The methods for culturing cell monolayers, labelling these cells and preparing DNA fibre autoradiographs have been outlined previously in the General Account of Materials and Methods. FUdR and uridine were applied, at the concentrations previously stated, to chick somatic cells for 16 hr. From whole cell autoradiography the cell synchrony achieved was calculated to be 33%. Thus a third of the cells synthesising DNA should have begun their S-phases when thymidine was made available in the form of ^{3}H-TdR. The specific activities of thymidine used ranged from 23 to 27 Ci/m Mol. Cells were labelled with ^{3}H-TdR for 30 min, the radioactive growth medium was then removed and replaced with non-radioactive growth medium for a further 30 min chase period. Cells were immediately harvested and resuspended in a concentrated sucrose solution to terminate labelling. DNA fibre autoradiographs were prepared according to the dialysis chamber method. Exposure times varied from 24 to 32 weeks.

Initiation intervals, and replicated lengths used to determine the degree of staggering in times of initiation between tandem replication units, were measured directly from the autoradiographs to ± 1 µm using an eyepiece scale. Photographs were taken at a magnification of x 100 and enlargements made x 5 so that the total magnification in all the photographs shown in Plates 8 to 22 is x 500.

Grain density readings were estimated from the photographs using a binocular microscope. The total magnification was x 8. Readings were made using an eyepiece scale. Estimates of grain density per unit length were made according to the number of divisions out of twenty on the scale which were filled by silver grains on the autoradiograph. Since the apparent number of silver grains varies inter alia as a result of fusion between grains, this method of measuring enabled a higher degree of accuracy than was possible using a direct grain count. Twenty divisions on the eyepiece scale equalled a length of 16 µm on the autoradiograph; each 1/20 division therefore corresponded to 0.8 µm.

RESULTS

(i) Fibre density

Plates 7a and 7b are low-power, dark ground illuminated photomicrographs of the distribution of silver grains in the overlying autoradiographic film from complete areas of two Millipore filters exposed to dialysis. The filter shown in 7a was taken from a dialysis chamber that had been filled with far too high a concentration of cells. Even when chambers are filled with that concentration of cells empirically established to produce the ideal density of DNA fibres required on the filter, great variations in fibre density still occur. Only in autoradiographs of filters or regions of filters where the

density of fibres is low is it possible to infer the presence of single extended DNA fibres and follow their pattern of replication. Plate 7b shows the low density required to visualize single fibres without ambiguity. From both the above photographs it can be seen that the silver grains appear in tracks. These tracks represent labelled lengths of extended single fibres of DNA. These fibres stretch out along the line of drainage to the pin-hole.

(ii) Tandem arrangement

Plates 8 to 22 are high-power photomicrographs showing the distribution of radioactivity in DNA from cells labelled for 30 min and chased for 30 min. Heavily labelled regions represent DNA replicated during the first 30 min pulse. The chase results in a progressive dilution of the radioactivity incorporated in DNA synthesized during the chase period, giving rise to regions of diminishing grain density of 'tails'. One main feature is apparent: labelled sections often occur in tandem, and therefore replication of chromosomal DNA must occur, at least to some extent, concurrently at many initiation sites arranged along a DNA fibre. The tandem relationship of several labelled tracks may be recognised with fair assurance in those regions of the autoradiographs where the line of drainage is even and the fibre density low. Here no confusion arises from labelled tracks of DNA fibres lying in parallel as to which members form a tandem sequence.

(iii) Directionality of replication

The direction of replication from initiation sites can be inferred from the spatial relationship between "later" tails and "earlier" heavily labelled DNA fibres. Huberman & Riggs (1966, 1968) and Huberman & Tsai (1973), have claimed that from most if not all initiation sites replication proceeds bidirectionally. Lark, Consigli & Tolliver (1971), on the contrary, have

claimed that "replication of mammalian DNA can proceed via a single replication fork at each replicon (replication unit) and that the direction of replication need not be the same for adjoining replicons in the same DNA fibre."

In the simplest situation, if replication is bidirectional from an initiation site which came into operation during the initial pulse labelling period and continued, without fusion with neighbouring sections during the later chase period, then a heavily labelled track flanked on both sides by tails should be evident in the autoradiograph. Likewise, if replication is unidirectional from an initiation site which came into operation during the initial pulse labelling period and continued, without fusion with a neighbour during the subsequent chase period, then a heavily labelled track flanked on only one side should be evident.

Before collecting information as to whether the direction of replication is generally bidirectional or unidirectional in chick somatic cells, the potential ambiguity of certain examples of labelled sections must be considered.

(1) Bidirectional replication may only be inferred with absolute assurance with regard to a middle or internal heavily labelled track within a tandem sequence of replication units carrying tails at both ends i.e. flanked on both sides by neighbouring replication sections as yet not confluent with the internal unit. A single heavily labelled track flanked on both sides by tails might conceivably result from the confluence of two units replicating unidirectionally, both in the same direction, with accidental breakage and loss of that part of one of the units which was replicated during the pulse. The same reasoning may be applied to two such heavily labelled tracks

in tandem and carrying tails at both sides. Such an accident is, however, unlikely to be frequent; that it should occur twice in juxtaposed regions must be extremely remote.

(2) Certain tracks will necessarily record the continuing replication of units which had already initiated before the pulse was applied. If replication is bidirectional one would anticipate finding pairs of heavily labelled tracks with single tails to the "outside" i.e. back-to-back, with an unlabelled gap in between. Such tracks are found. They might, however, conceivably be interpreted as the outcome of unidirectional replicating units, with the gap representing not DNA already replicated before the pulse, but rather as yet unreplicated DNA. Although this consideration is indeed valid, such internal divergently labelled tracks are taken to be one-way sections of a bidirectional replication unit in the present study for the following reasons. First, such tracks regularly occur back-to-back; if the proposals of Lark et al. for unidirectional replication are correct a random arrangement of such one-way sections would be expected. Secondly, such tracks are always of approximately equal length; this would be the expected situation were two growing points to proceed at equal rates in opposite directions from a shared initiation site without fusion. Thirdly, the unlabelled region at the centre of two divergently labelled tracks is considered to be already replicated DNA because, where sister strand separation of fully replicated regions of DNA occurs, as for example in Plates 11, and 20, the unlabelled regions form part of the separated and therefore fully replicated sister strands.

(3) For reasons similar to those outlined in (1) and (2) above, unidirectional replication may only be inferred with firm assurance in regard to a

heavily labelled section carrying a tail at one end, flanked to the inside of its initiation site by a neighbouring replication unit carrying a facing tail, both replication units being unfused.

The diagrams in Fig. 4 show examples of the patterns of labelled sections used in the present study to infer the direction of replication: (a) and (b) show the arrangement of labelled sections required as firm evidence for bidirectional replication in the labelled sections indicated by arrows; (c) and (d) the pattern required to infer unidirectional replication in the labelled sections indicated by arrows.

Following the challenge to bidirectional replication made by Lark et al. in 1971, Huberman & Tsai, in a recent paper in 1973, attempted to confirm that replication of eukaryotic DNA was indeed bidirectional. For these reasons the potential ambiguity as to the direction of replication of certain types of labelled sections has been dealt with more thoroughly.

Fig. 5 is taken from Huberman & Tsai (1973) and is their Fig. 1 (with reference to their High-Low specific activity shift). It shows what they regard to be patterns of grains on autoradiographs that may be interpreted as having resulted from replication of DNA in a bidirectional manner from two initiation sites (2, 3) or alternatively replication in a unidirectional manner (1). Fig. 6 is also taken from the same reference and is their Fig. 2. This shows examples of grain patterns which they consider ambiguous as regards whether replication is bidirectional or unidirectional.

Taking into account all the problems outlined above, however, not only are the diagrams in Fig. 6 rightly ambiguous but also those indicated in Fig. 5. In Fig. 5 (1) unidirectional replication cannot be inferred for the R-hand labelled section because such an example may also be considered

Fig. 4. The minimum number of labelled tracks in tandem with 'tails' used in the present study of DNA fibre autoradiographs from pulse-chased cells to differentiate unambiguously between bidirectional and unidirectional replication. (a) and (b) illustrate the arrangement of heavily-labelled tracks (solid lines), tails (broken lines) and unlabelled gaps indicative of bidirectional replication while (c) and (d) illustrate the arrangement of heavily labelled tracks and tails indicative of unidirectional replication.

Fig. 5. Adapted from Huberman & Tsai (1973) and is their Fig. 1, with reference to a 'High to Low' specific activity shift. According to Huberman & Tsai these represent examples of unambiguous autoradiographic patterns. The dotted line indicates unlabelled DNA, the solid line indicates DNA labelled at high specific activity, and the broken line indicates DNA labelled at low specific activity. The strand diagrams are intended to provide a reasonable explanation of the types of autoradiographic patterns illustrated.

Fig. 6. Copied from Huberman & Tsai (1973) and is their Fig. 2. According to Huberman & Tsai these show examples of ambiguous autoradiographic patterns. The solid lines indicate regions of high grain density and the broken lines indicate regions of low grain density. These patterns, they state, are ambiguous either because there is no way of telling whether they end due to breakage of the DNA molecule or due to termination of labelling ((a), (b), (c)) or because no contiguous grain density shifts are visible ((d), (e), (f)).

Fig 4

Fig 5

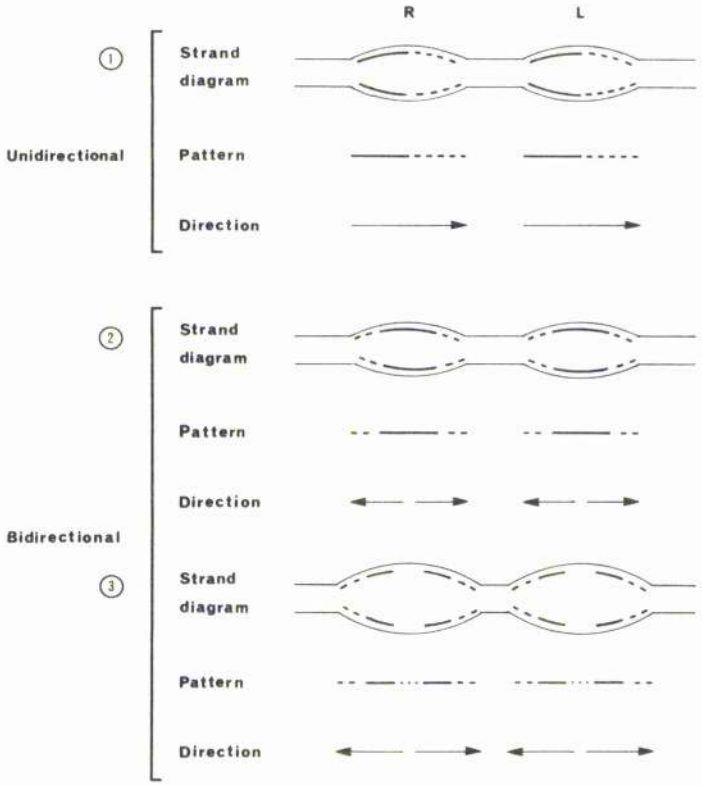


Fig 6

as a one-way section from a bidirectional replication unit that initiated at some distance away before label was available. Bidirectional replication cannot be inferred with absolute assurance for either labelled section represented in Fig. 5 (2) because neither are internal with respect to neighbouring replication units. Likewise, bidirectional replication cannot be concluded in Fig. 5 (3) because both fusion and breakage of the DNA at the free ends cannot be excluded.

Turning now to the interpretation of those labelled sections observed in autoradiographs from chick somatic cells; bidirectional replication may be inferred in the majority of cases, see Plates 8 to 22. Unidirectional replication could only be attributed to a very few labelled sections. One such example is shown in Plate 8, the labelled section carrying a diverging tail second in from the right end of the tandem sequence. Such examples might be unambiguous, but because I think it is more likely that one pattern of replication occurs within eukaryotes generally and because the overwhelming majority of examples in these autoradiographs are definitely bidirectional in pattern, such examples of peculiar and exceptional tracks I interpret as being occasional accidents so characteristic of autoradiography! Having taken bidirectional replication as the rule, ambiguous tracks are interpreted according to a bidirectional model. The end sections of a tandem series showing a single internal tail, as for example in the rightmost tracks in Plates 8, 12 and 22, are thought to be one-way sections of a replication unit that had initiated some distance away before label was available. Sometimes fusion of two converging one-way sections, from neighbouring initiation points that had begun replication before the label was provided, results in a region of low grain density being flanked on both sides by heavily labelled regions as in Plates 10 and 14. In some cases heavily-labelled sections may

carry no tails; here fusion may have occurred between two converging one-way sections prior to the chase period, as in Plate 16 and 17.

(iv) Staggering of initiations in time

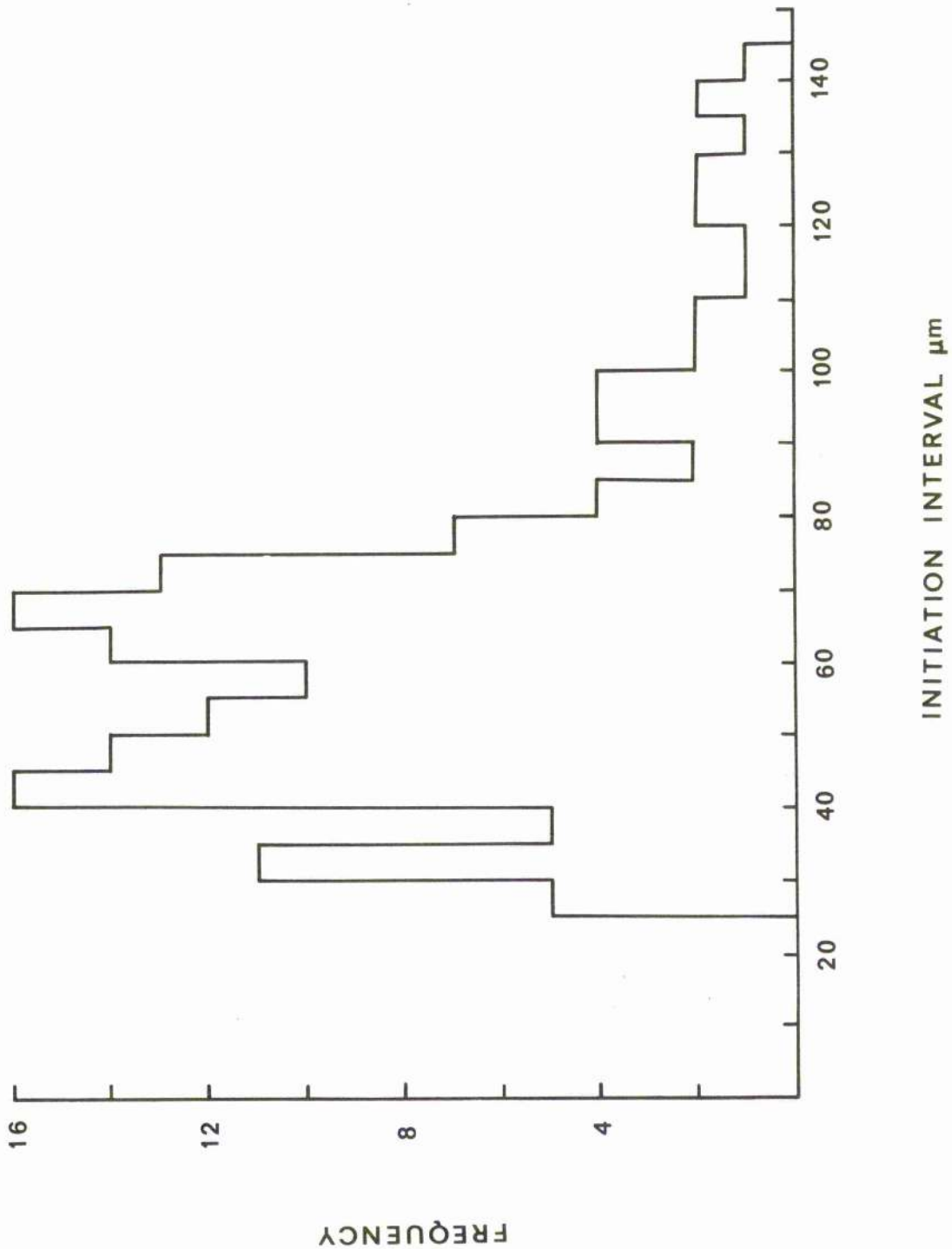
From all the above observations it is evident that within a tandem series of replication units, initiations may not be synchronous; the degree of asynchrony is examined more closely on p. 50. In such a tandem series a pair of one-way sections may flank a long mid-gap whilst neighbouring replication sections may show either a short heavily-labelled length carrying tails on both sides, or a long heavily-labelled length again carrying tails on both sides. Assuming a constant rate of replication, the first mentioned replication unit must have initiated some time before label was applied whereas its neighbours initiated after label was available, the unit showing the longer labelled length having initiated first. Plates 11, 19, and 21 show examples of these different combinations.

(v) Determination of initiation intervals

The distance between neighbouring initiation points is called the initiation interval. This interval was measured as the mid-point to mid-point distance between neighbouring heavily labelled sections, either uninterrupted or with an unlabelled mid-gap, showing tails on both sides. Fig. 7 shows the frequency distribution of initiation intervals measured in DNA fibre autoradiographs from cells pre-treated with FUdR for 16 hr, labelled for 30 min and chased for 30 min. 150 measurements were recorded. The mean initiation interval is 63 μm and the range is 25 to 145 μm. The mean initiation interval between replication units also gives an estimate of the mean replication unit length. The main feature that is apparent, however, is that

Fig. 7. Frequency distribution of initiation intervals from DNA fibre autoradiographs derived from chick somatic cells in culture. The cells were FUdR-treated, labelled with ^{3}H-TdR for 30 min, and 'chased' for 30 min.

Fig 7


FREQUENCY
16
12
8
4
20
40
60
80
100
120
140
INITIATION INTERVAL µm

initiation intervals may differ in length; on p.47 the extent of this variation within tandem sequences is examined more closely.

The following sources of error which occur when collecting this data must now be considered.

(1) Initiation points lying particularly close together would be overlooked if fusion of the neighbouring replication units had occurred within the first 30 min of labelling.

(2) Some measurements would overestimate initiation intervals if such already-fused replication units (as in 1) were taken to be single units.

(3) Some apparent intervals may in fact encompass initiation sites which would have begun replication more than one hour after the start of labelling.

(4) The longer the initiation interval the greater the possibility of shear breakage occurring, thereby destroying the tandem relationship.

(5) The longer the initiation interval the greater the possibility of not relating two genuine neighbouring replication units.

Errors accruing from sources 1 to 3 tend to bias the data in favour of longer initiation intervals, while errors from sources 4 and 5 do the reverse.

(vi) Interpretation of tandem arrays

Fig. 8 shows the interpretation of two tandem sequences of replication units; Fig. 8 (i) concerns Plate 11 which shows sister strand separation, and 8 (ii) concerns Plate 15, where no separation is apparent. In Fig. 8 (i) the pattern of heavily labelled regions and tails observed from the

Fig. 8. An interpretation of the replication pattern along short regions of two DNA molecules from the arrangement of tandemly labelled sections of nascent DNA, as observed in DNA fibre autoradiographs prepared from chick somatic cells, pre-treated with FUdR and labelled for 30 min and chased for 30 min. Thin continuous lines represent parent polynucleotide chains, dotted lines indicate nascent DNA replicated prior to the addition of ^{3}H-TdR, heavy continuous lines indicate nascent DNA replicated during the first 30 min pulse and heavy broken lines indicate nascent DNA replicated during the subsequent 30 min chase. O, represents an origin or initiation site.

i. Redrawn from Plate 11 and showing sister strand separation.

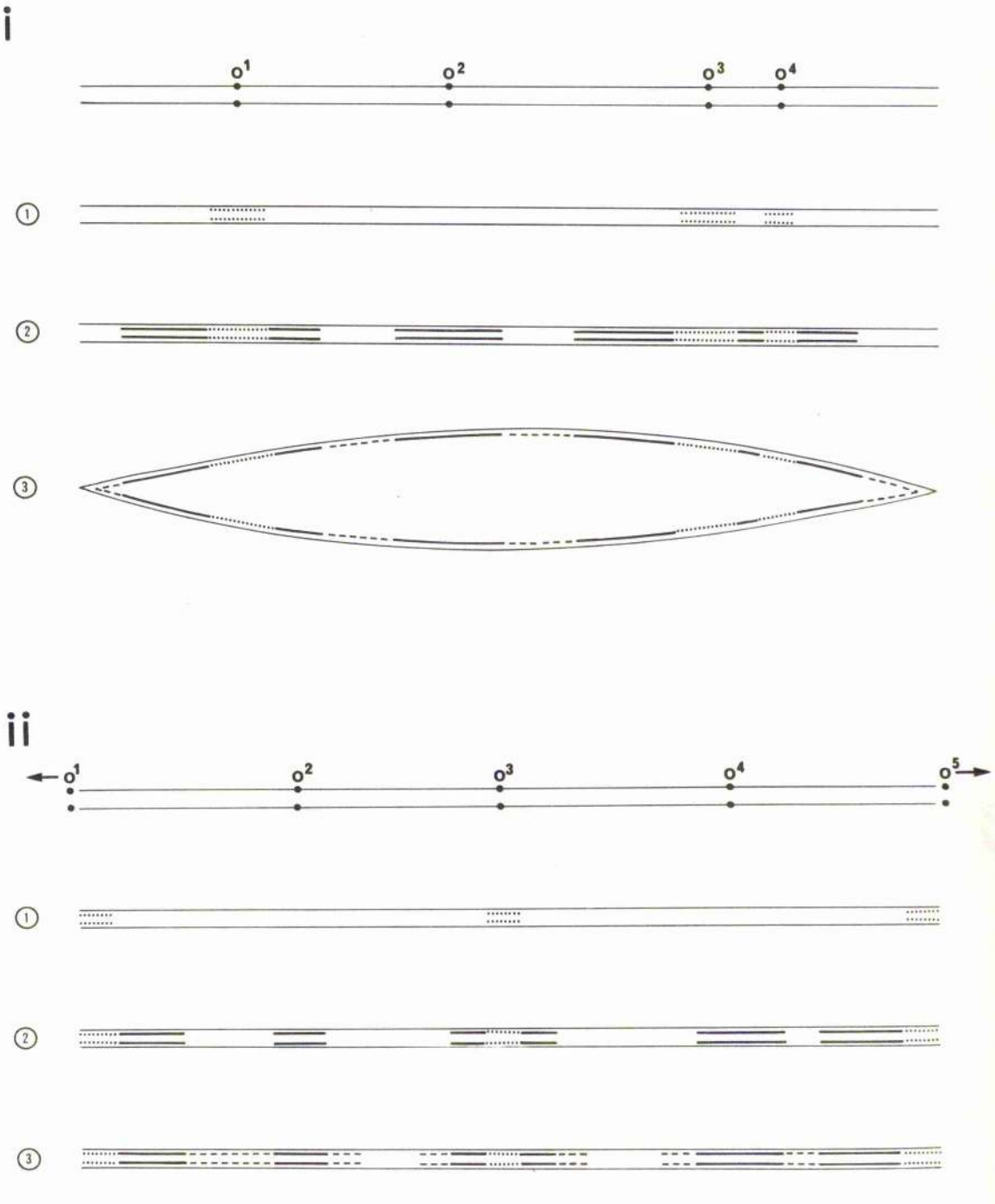
ii. Redrawn from Plate 15, no separation of sister strands is apparent.

(1) Pattern of replication prior to labelling.

(2) Pattern of replication that would have been observed if cells were harvested after the first 30 min pulse.

(3) Patterns of replication as observed in the Plates and following 30 min labelling and 30 min chase periods.

Fig 8



autoradiograph and redrawn in (i) 3, suggest origins at positions O_1, O_2, O_3 and O_4. It may be inferred from the unlabelled mid-gaps in the tandem array that origins O_1, O_3 and O_4 initiated prior to the availability of ^{3}H-TdR, (i) 1. During the 30 min period of labelling, (i) 2, origin O_2 must have initiated and DNA synthesis continued from all origins in a bidirectional manner along both parent DNA strands. Fusion of the converging one-way sections from origins O_3 and O_4 must have occurred early in this period, resulting in the short heavily labelled section flanked on either side by unlabelled regions. During the subsequent chase, (i) 3, fusion occurred between the converging tails of neighbouring replication units that had initiated at positions O_1, O_2 and O_3. Similarly in Fig. 8 (ii) origins are inferred at positions O_1, O_2, O_3, O_4 and O_5, although origins O_1 and O_5 cannot be located. Prior to labelling (ii) 1, initiation must have occurred at origin O_3 and presumably some distance away at origins O_1 and O_5, although shear breakage of the labelled DNA could also have produced this pattern of end one-way sections. During the 30 min labelling period, (ii) 2, initiation occurred at origins O_2 and O_4. During the chase period, (ii) 3, fusion must have taken place between converging one-way sections from origins O_1, O_2, O_4 and O_5. When the cells were harvested fusion had not yet taken place between converging tails from either origins O_2 and O_3 or from origins O_3 and O_4.

These examples provide models for the analyses of most of the fibre autoradiographs shown in Plates 8 to 22. Such analysis is necessary to determine origins, to measure initiation intervals, to determine the variation in initiation intervals within a tandem sequence, the degree of staggering of initiations in time and the variation of staggering within a tandem sequence.

TABLE 1. RELATIVE GRAIN DENSITY

(Number of 1/20 divisions filled over 16 μm)

Single track	Separated sister strand	Ratio
15	8	1.87
16	11	1.36
12	5	2.40
11	4	2.75
15	7	2.14
16	8	2.00
13	6	2.16
16	7	2.28
11	7	1.57
14	5	2.80
	Total =	21.33
	∴ Mean =	2.13

(vii) Separated sister strands and fork-like growing points

A precise corresponding pattern of label distribution along two strands lying side by side as in Plate 11 is indicative of completely replicated and separated sister strands of DNA. From such patterns the fork-like progression of replication can be inferred, because radioactivity is evidently being incorporated concurrently into both daughter strands of DNA. Fully replicated lengths of DNA can only occasionally be seen as separated sister strands, and usually only when a long length of DNA has been replicated. Although replication occurs concurrently alongside both parental polynucleotide chains, if the two sister double helices remain in close juxtaposition the resulting autoradiograpy necessarily shows only a single track of grains. Evidence that single labelled tracks of silver grains are usually the outcome of radioactivity incroporated into two sister double helices is provided by the fact that the grain density resulting from separated sister strands is about half that found in most single labelled tracks. See for example Plates 10, 11 and 20. Table 1 compares the density of silver grains in 16 mm lengths (20 divisions on the eyepiece scale) from labelled, separated sister strands and neighbouring single tracks in tandem series. The mean ratio is 1 : 2.13. Comparisons were always made within the same tandem sequence because the intensity of silver grains may vary widely between different regions of a single autoradiograph.

(viii) Is the length of neighbouring intervals similar?

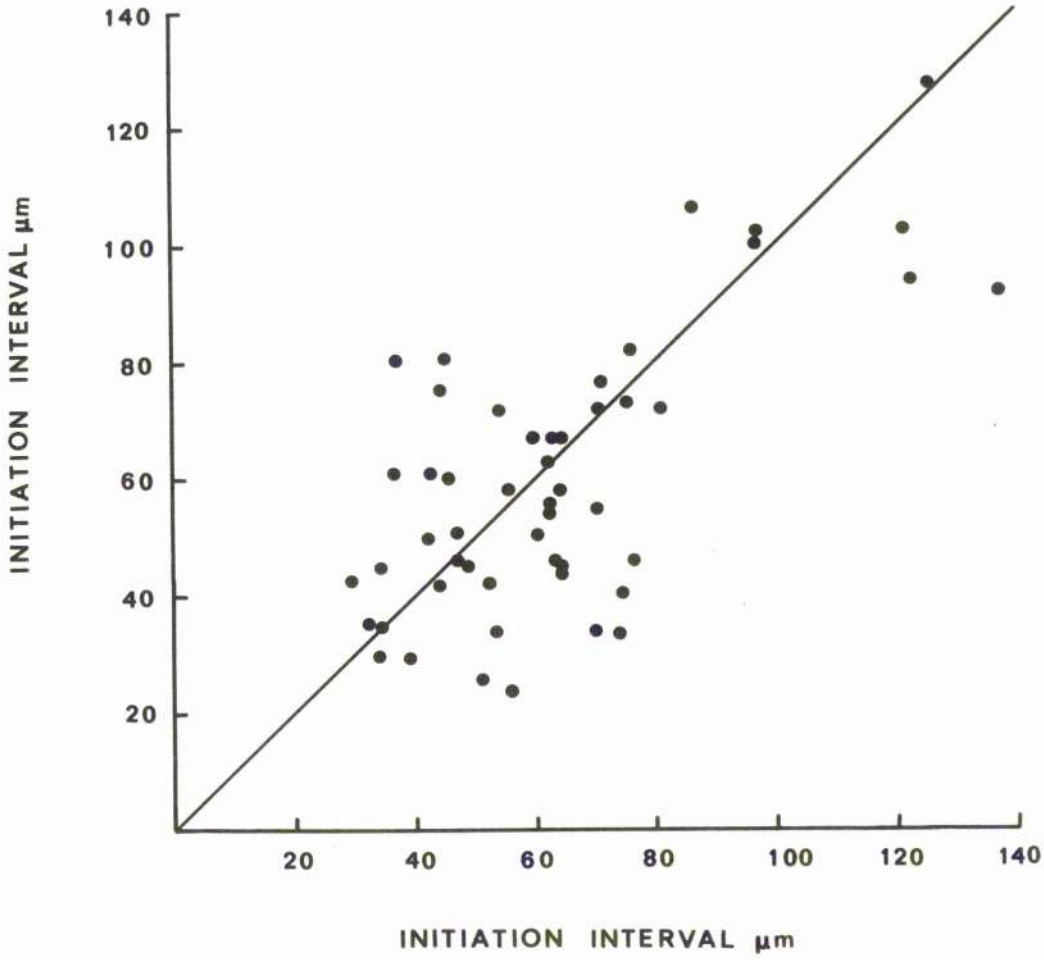
It will be recalled that initiation intervals range from 25 to 145 µm. A question arises as to whether, within the genome of the chicken, the length relationships between neighbouring intervals are random or whether intervals of similar length tend to occur together i.e. do short lengths march with

short lengths and long with long? Fig. 9 is a correlation plot of 50 randomly selected pairs of neighbouring initiation intervals, each pair being taken from a separate tandem series. The first member (x) of each pair is plotted along the ordinate and its second member (y) along the abscissa. A mid-line is drawn between ordinate and abscissa to represent the predicted values of (x) and (y) were they to be the same length in each pair. The deviation, a vertical line connecting the observed value with predicted value would represent the difference between a pair of measurements (not drawn). This correlation may also be tested statistically, using the same results as shown on the graph.

The question initially asked was: 'do short x's occur with short y's, and long x's with long y's?' A rephrasing of this question would be: 'Is there more variability between pairs than within pairs?' This question is a standard question to which a standard statistical answer may be given by an analysis of variance. The hypotheses that are tested by means of the analysis of variance are whether the means of several populations are equal. Prior assumptions must be made in this test: (1) all observations are random samples from Normal distributions; and (2) the Normal populations all have equal variances. Thus all measurements of (x) and (y) are converted to logarithms before analysis to make distributions more nearly Normal. For the purposes of this test it must first be established that there is no special correspondence between the first members (x) of the pairs nor between the second members (y). Then with (p) pairs there are two contributions to each measurement, one dependent on whether it is the first or second member of the pair, the other dependent on which particular pair is measured - together with a residual natural variation. The procedure for analysis will now be outlined briefly.

Fig. 9. Graph showing the correlation in length within 50 pairs of neighbouring initiation intervals. Intervals were measured from DNA fibre autoradiographs prepared from FUdR-treated chick somatic cells labelled with ^{3}H-TdR for 30 min and chased for 30 min.

Fig 9


140
120
100
80
60
40
20
INITIATION INTERVAL µm
20
40
60
80
100
120
140
INITIATION INTERVAL µm

(1) Formulate the null and alternative hypothesis:

Ho : the means of the values (x) and (y) are equal.

against H 1 : the means of the values (x) and (y) are not equal.

Ho : the means of the values 'between pairs' and 'within pairs' are equal.

against H i : the means of the values 'between pairs' and ' within pairs' are not equal.

A level of significance is then decided upon. ($\alpha = 0.01$)

(2) A table is constructed:

	Measurement x	Measurement y	Total
Pair 1	x_1	y_1	z_1 $(x_1 + y_1)$
Pair 2	x_2	y_2	z_2 $(x_2 + y_2)$
.	.	.	.
.	.	.	.
.	.	.	.
p	xp	yp	zp (xp + yp)
Total	X	Y	Z

(3) From this an analysis of variance table is constructed from the following calculations:

(a) Square and Add $x_1^2 + x_2^2 + \ldots\ldots xp^2$

(b) Square and Add $y_1^2 + y_2^2 + \ldots\ldots yp^2$

(c) Square and Add $(x_1 + y_1) = Z_1^2$, $(x_2 + y_2) = Z_2^2$, $\ldots\ldots$ $(xp + yp) = Zp^2$

(See Table 2)

TABLE 2. Analysis of variance (a) between first members of each pair, and second members of each pair, and (b) between individuals of each pair.

Source of variation	Degrees of freedom	Sum of squares	Mean squares
Between x and y	(1)	$\frac{X^2 + Y^2}{p} - \frac{Z^2}{2p} = 0.00966$	0.00966 (M_1)
Between individuals	(p - 1) = 49	$\frac{1}{2}(x_1^2 + x_2^2 + \ldots + x_p^2) - (Z^2/2p)$ = 2.06219	0.04209 (M_2)
Residual	(p - 1) = 49	by subtraction = 0.50342	0.01027 (M_3)
Total	(2p - 1)	$x_1^2 + x_2^2 + \ldots + x_p^2 + y_1^2 + y_2^2 + \ldots y_p^2 - (Z^2/2p)$ = 2.57527	

(a) $(M1/M3)F = 0.94$, $F_{0.01} = 7.19$

(b) $(M2/M3)F = 4.09$, $F_{0.01} = 1.9$

p = No. of pairs

x = First member of pair

y = Second member of pair

X = Total $x_1 + x_2 + \ldots + x_p$

Y = Total $y_1 + y_2 + \ldots + y_p$

Z = X + Y

z = x + y, ($z_1 = x_1 + y_1$, $z_2 = x_2 + y_2$, $\ldots$ $z_p = x_p + y_p$)

(4) The analysis of variance table is completed and in each line, except the total, a Mean Square is calculated by dividing the Sum of Squares by its degree of freedom (d.f.) M_1, M_2, M_3.

(5) To test whether there is a consistent difference between the first (x) and second (y) measurements the ratio $\frac{M_1}{M_3}$ is compared with tabulated values of the F-statistic with (1) and (p-1) degrees of freedom.
If $F < F_{0.01}$, accept Ho : the means of the values (x) and (y) are equal.
Since $F, \frac{M_1}{M_3} = \frac{0.00966}{0.01027} = 0.94$, $F < F_{0.01}$ (7.19), accept Ho.

(6) The test which answers the original question 'Is there more variability between pairs than within pairs,' is to compare the mean squares $\frac{M_2}{M_3}$ with tabulated values of the F-statistic with (p-1) and (p-1) degrees of freedom. If $F > F_{0.01}$ reject Ho : the means of the values 'between pairs' and 'within pairs' are equal. $F_1 \frac{M_1}{M_3} = \frac{0.04209}{0.01027} = 4.0$, $F > F_{0.01}$ (1.9). Therefore reject Ho.

It is concluded that a tendency does exist for like initiation intervals to occur alongside each other. This tendency may be directly observed in tandem sequences in Plates 12, 14, and 15.

(ix) <u>Is the activation of neighbouring origins near synchronous?</u>

A question remaining is whether, within a length of chromosomal DNA there is or is not a tendency for neighbouring initiation sites (origins) to come into operation synchronously. We already know that there can be great staggering between immediately adjacent initiation sites (see Plate 20) but such staggering in time of operation might be a feature of <u>borders</u> between groups or clusters while within groups there might be synchronous or near-synchronous starts. Therefore the question is effectively: is there any evidence for a

clustering of initiation sites which come into synchronous or near-synchronous operation?

To measure the time over which a certain replication unit has been replicating two assumptions must be made. First, that termini do not exist. This proposal is discussed on p. 73 and it is concluded that the assumption is indeed valid. Secondly, that replication rate is invariant. This is discussed on Chapter 3 where although no definite answer is available the assumption is considered reasonable.

The degree of staggering in initiation times is measured for neighbouring replication units, each pair taken from a separate tandem series, from the total length of DNA that has been replicated in each unfused replication unit up to harvesting of the cells. Replication units with long mid-gaps would result in long lengths being recorded, unfused replication units that initiated immediately label was applied and continued replication during the 30 min period of labelling and 30 min chase would have labelled lengths equal to the amount of DNA synthesized over 60 min and short labelled lengths would result from initiation of DNA synthesis sometime after ^{3}H-TdR was supplied.

To enable recognition of initiation points and therefore the lengths of DNA synthesized from each origin, such measurements must be recorded from chased preparations. However, two problems arise. First, tails do not have precise limits and secondly, fusion frequently occurs at the tails of adjacent replication units after a 30 min labelling and 30 min chase period. See for example Plates 9, 12 and 19. Length measurements were therefore only made of heavily labelled tracks excluding the tail regions. A certain error must occur in the evaluation of the point at which a heavily labelled region ends and a tail begins. This error is borne in mind.

Fig. 10. Graph showing the degree of synchrony in the time of initiation of neighbouring replication units. Interpreted from the total replicated length (heavily labelled track plus mid-gap, if present) excluding tails, of 25 pairs of neighbouring and unfused replication units from DNA fibre autoradiographs prepared from chick somatic cells, pre-treated with FUdR, labelled with ^{3}H-TdR for 30 min and chased for 30 min.

Fig 10

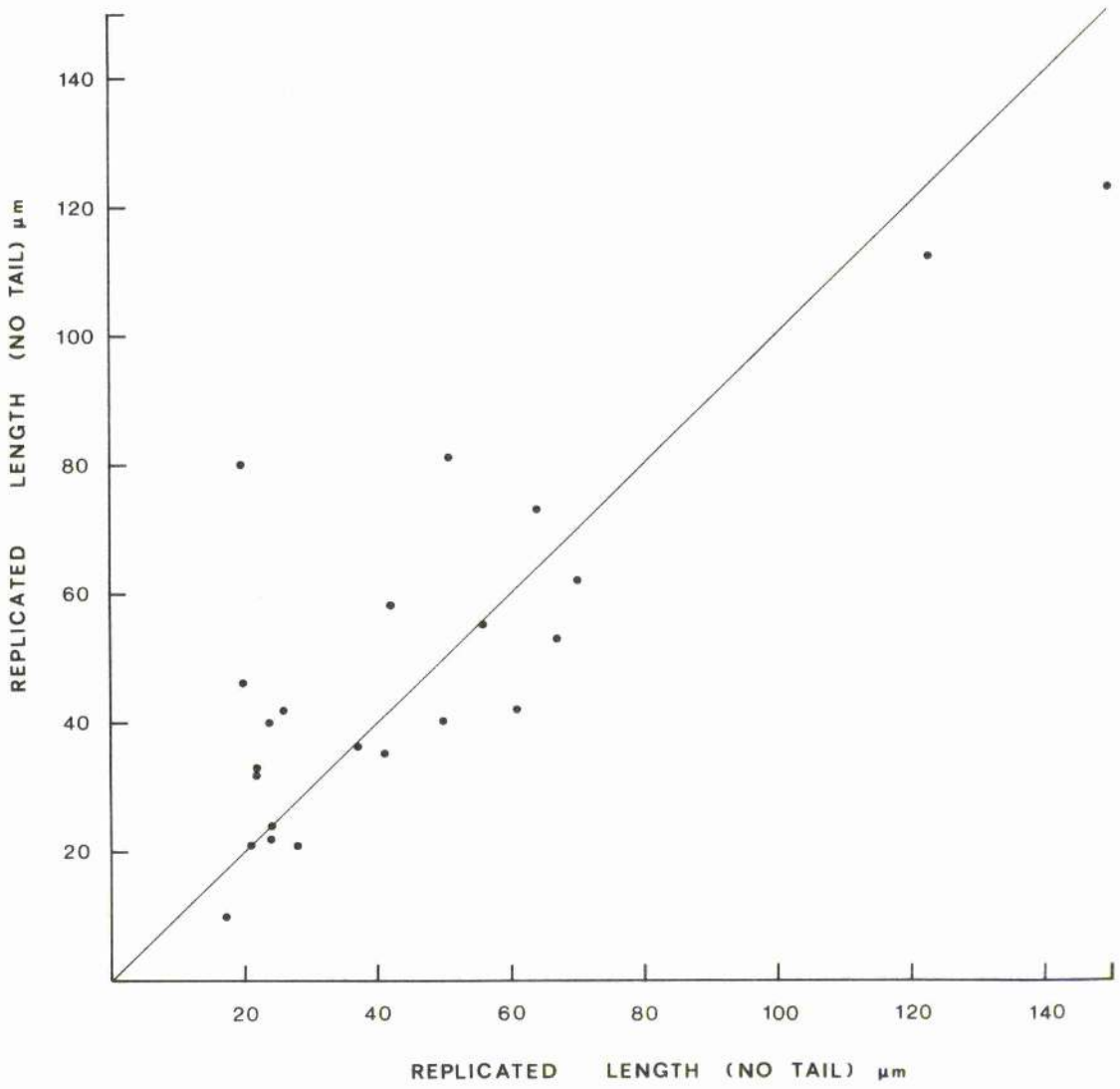

140
120
100
80
60
40
20
REPLICATED LENGTH (NO TAIL) µm
20
40
60
80
100
120
140
REPLICATED LENGTH (NO TAIL) µm

Fig. 10 is a correlation plot of replicated lengths from 23 pairs of neighbouring unfused replication units, each pair taken from a separate tandem series. As in Fig. 9, the mid-line in Fig. 10 represents the predicted values of the two measurements from each pair were they to be equal. The deviation of the observed value from the mid-line represents the variation in replicated lengths between members of a pair i.e. the variation in the times of initiation between two adjacent replication units.

Because of the problems outlined above only a small number of pairs were measured. A valid statistical analysis of such a small number of results was not possible. However, it can be seen from the graph that the distribution of points is not random but shows a tendency for points to fall near the mid-line. It may be concluded therefore that a tendency does exist for neighbouring replication units to initiate at relatively similar times. Where neighbouring replication units were recorded which initiated at greatly staggered times, examples of the previously referred to "border pairs" may have been measured.

DISCUSSION

(i) Method of "chase"

A possible criticism of chase experiments carried out in the way described in this chapter i.e. an initial period of high intensity labelling followed by a period of further growth in unlabelled medium, is that when ^{3}H-TdR enters the cell it passes into an already existing pool of DNA precursors. Depending on the rate of entry and pool size, the specific activity of DNA synthesized at the beginning of labelling would be expected to rise, slowly or rapidly, until the specific activity of precursor molecules had risen to equilibrium. This effect would appear in autoradiographs

as a grain gradient at the beginning of labelling, and might be misconstrued as a tail consequent upon the chase. That this source of error is not serious is evident in certain tracks from chased material to be seen in many labelled sections shown in Plates 8 to 22. Origins which initiated replication some time before labelling started, and which therefore show up in autoradiographs as gaps flanked by heavily labelled tracks, have well defined distal tails but their proximal heavily labelled regions begin abruptly. It follows that the size of the pool of thymidylate molecules must be sufficiently small, at least after FUdR treatment, to allow rapid swamping by the influx of ^{3}H-TdR at the beginning of the pulse. On the other hand the pool must be sufficiently large to allow the autoradiographic detection of the progressive dilution of the remaining ^{3}H-TdR during the later chase.

(ii) Bidirectional replication

A growing fork has been defined as any one site on a parental double stranded DNA molecule where replication has resulted in the production of two daughter strands of DNA. If two divergently arranged growing forks are produced from an initiation site, then two polymerase molecules must clamp onto the initiation site as a pair and start replication simultaneously. It therefore seems reasonable that initiation sites might be regions along the DNA where there are reversed complementary base sequences i.e. palindromes, the two polymerase molecules clamping onto the initiation site at the border between the two complementary sequences.

Huberman & Riggs DNA fibre autoradiographic studies on Chinese hamster cells in 1968 showed central regions of high grain density bounded at both ends by gradients of declining grain density when an initial pulse

and later chase procedure, similar to that used in the present study, was employed. They were led to propose that synthesis of the DNA had been proceeding bidirectionally from a common origin in the great majority of cases. My own evidence for chicken somatic cells confirms this pattern.

Lark, et al. (1971) again working with FUdR-treated Chinese hamster cells but using a step-up labelling procedure i.e. a pulse of low specific acitvity label, 5.1 Ci/mMol for 30 min followed by a pulse of high specific activity, 11.9 Ci/mMol for 45 min, claimed that the majority of the labelled sections they examined showed unidirectional replication. When however they did pulse-chase experiments of the type reported by Huberman & Riggs and myself, nearly all the fibres they examined showed bidirectional replication. They do not attempt to answer this anomaly.

In reference to their first experiment it will be recalled that once replication has progressed some distance from an initiation point in the absence of label, any single fork will necessarily appear to be replicating one-way! Again, if the difference in specific activities of the Low - High pulse-chase protocol was not sufficient to produce a demonstrable increase in grain density during the chase period, their conclusion as to the direction of replication based on their interpretations of which labelled sections represented tail regions, must be considered unsound. This has also recently been investigated by Huberman & Tsai (1973). In the light of Lark et al's evidence they re-examined their proposal for bidirectional replication, and repeated DNA fibre autoradiographic experiments using various labelling protocols, including an exact repeat of Lark et al's first experiment. They concluded that about 90% of their unambiguous autoradiographic patterns could be explained by bidirectional replication, not by unidirectional replication. They also found that in autoradiographic experiments using two different specific activities of ^{3}H-TdR, obvious

differences in grain density were obtained only when the difference in specific activity was threefold or more. The two-fold difference in specific activity employed in experiments by Lark et al. referred to earlier was therefore not adequate to allow a difference in grain density to be observed. In addition to the evidence from the present study and that presented by Huberman and co-workers, DNA autoradiographic patterns consistent with bidirectional replication in eukaryotes have also been observed by Callan (1972, 1973), using both the pulse-chase and the step-up labelling procedure (see notably Fig. C, Callan, 1973) and by Hand & Tamm (1973) and Amaldi et al. (1972) using a pulse-chase protocol. Furthermore Weintraub (1972a) detected bidirectional replication in chicken somatic cells by an entirely different technique based on specific breakage of 5-bromouracil (BrdU) - containing DNA by ultra violet light. Synchronized cells were labelled with BrdU for 5 min and then with ^{3}H-TdR for 15 min. Similar control cells were labelled for 20 min with ^{14}C-thymidine. DNA was extracted from both and centrifuged through an alkaline sucrose gradient after exposure to ultra violet light. After 10 minutes irradiation the DNA containing BrdU was reduced to about one-half the molecular weight of the marker ^{14}C-DNA which was unaffected by irradiation. From these experiments Weintraub concluded that the BrdU segment was in the middle of the pulse labelled DNA and that replication was bidirectional at each replication unit.

Criticisms that FUdR pre-treatment might be responsible for an unnatural pattern of bidirectional replication, possibly due to a build-up of initiator proteins during FUdR treatment (Lark et al., 1971; Weintraub, 1972a) were tested in further experiments described in Chapter 4. These experiments demonstrate that labelled sections from non-synchronized, non-FUdR treated cells show at least the same degree of unambiguous bidirectional replication as DNA fibre autoradiographs prepared from FUdR-treated cells.

Finally, further support for the general occurrence of bidirectional replication may be taken from the evidence gained from prokaryotes. Bidirectional replication has now been found in phage λ (Schnös & Inman, 1970), E. coli (Bird et al., 1972; Masters & Broda, 1971), phage T4 (Delius et al., 1971), Salmonella typhimurium (Nishioka & Eisenstork, 1970) phage T7 (Wolfson et al., 1972) and in Bacillus subtilus (Wake, 1972).

(iii) Sister strands replicate concurrently

The present study also supports observations from other workers that at any one growing point both newly synthesized strands appear to extend in the same direction alongside the strands of the original duplex. Plates 10, 11 and 20, show the identical pattern of label distribution that is indicative of separated sister strands. That the grain density per unit length of supposed separated sister strands is indeed half that present in single labelled tracks from the same tandem series is shown in Table 1.

However, in vitro studies with DNA polymerases from both prokaryote and eukaryote sources have shown these enzymes are only capable of adding nucleotides to a pre-existing nucleotide chain in the 5' to 3' direction. Since the duplex strands of DNA are anti-parallel, how is the apparent concurrent replication of both parental strands from an initiation site to be explained? Recent evidence has accumulated in support of a model circumventing this difficulty first outlined by Okazaki, Okazaki, Sakabe, Sugimoto & Sugino in 1968. From experimental evidence on E. coli they proposed that DNA is in part replicated by a discontinuous mechanism involving synthesis and joining ('ligasing') of short deoxynucleotide units, each about 0.4 μm in length. Britten & Davidson (1971) Davidson et al. (1972) working with

Xenopus reported similar lengths of "unique sequence" DNA separated by short pieces of repetitious DNA. Evenson, et al. (1972) also discovered sections of this length, or multiples of it, regularly separated by short regions of low melting point, in DNA from Chinese hamster cells and chick fibroblasts. All this evidence points to the general and characteristic occurrence of these short sections of DNA (Okazaki fragments).

DNA synthesis occurs therefore in the 5' to 3' direction along one strand, either continuously or discontinuously, while on the complementary strand DNA is synthesised discontinuously again in 5' to 3' direction but necessarily away from the growing point. The short chains are then joined to the growing polynucleotide chain by formation of phosphodiester linkages catalysed by DNA ligases. The work of Pauling & Hamm (1969) suggests that replication is discontinuous along both sister strands of DNA. EM studies by Kriegstein & Hogness (1974) show that the direction of synthesis along sister strands is indeed antiparallel. Small "blip" regions along a stretch of DNA represent regions where DNA replication is occurring. In favourable circumstances a short length of single stranded DNA (representing unapired newly synthesised DNA) is observed at either end of a "blip" region, each extending from the opposite daughter strand of DNA. Recent work by Sugino et al. (1972) suggest that Okazaki fragments are initiated from short pieces of RNA transcribed as "fold-back" points in the separated parental DNA chains.

The above model is illustrated in its simplest form in Fig. 11 (1). Discontinuous replication is indicated along both complementary strands. An extension of this model must be proposed to explain bidirectional replication: such a model is illustrated in Fig. 11 (b). The synthesis of DNA can only occur in the 5' to 3' direction therefore bidirectional

Fig. 11. Models illustrating a simple 'high resolution' pattern of DNA replication. Short arrowed lines represent Okasaki fragments, O, represents the position of an initiation site or origin and solid continuous lines represent parent polynucleotide chains.

(a) Replication occurring unidirectionally along both complementary strands of DNA, discontinuously away from the initiation site in the 5' to 3' direction along one strand and towards the initiation site, again in the 5' to 3' direction, along the complementary strand.

(b) Bidirectional replication. DNA is replicated discontinuously away from the initiation site in the 5' to 3' direction along complementary strands. DNA is then replicated, again discontinuously in the 5' to 3' direction, along both complementary strands and towards the initiation site.

i

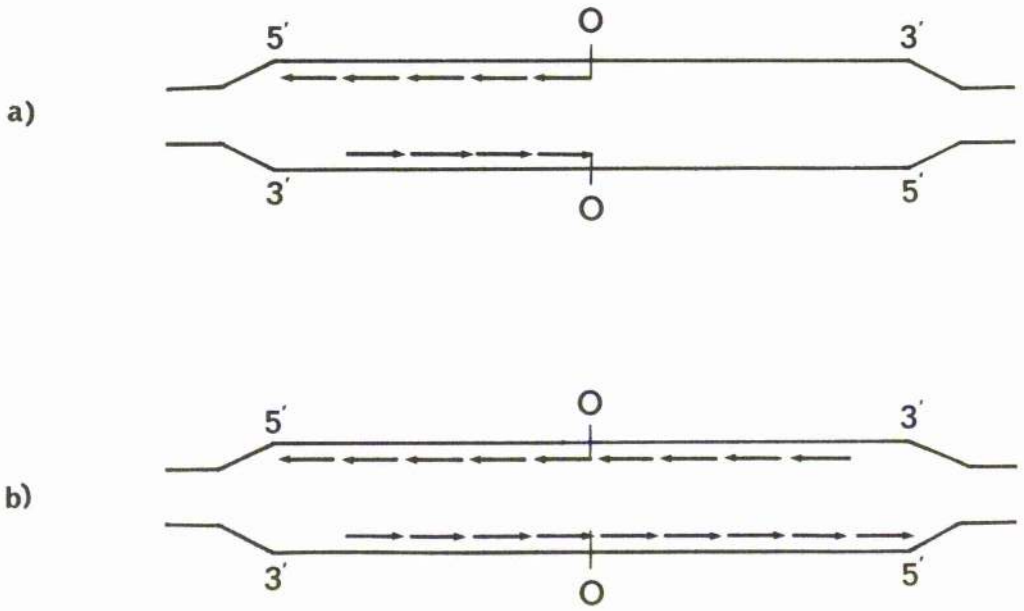


Fig 11

TABLE 3.

DNA REPLICATION IN EUKARYOTES

Cell type	C-value $g \times 10^{-12}$	S-phase duration hr	Temp. ^{o}C	Rate of DNA synthesis μm/hr one-way	Intervals between initiation points	Mean initiation interval
Xenopus	3	12	25	9	15 - 80	57
somatic t.c. (C.)						
Triturus						
somatic t.c.	29	48	25	20	100 - ?	200?
blastula	"	1	18	?	?	?
neurula	"	4 - 6	18	6	20 - 50	43
spermatocyte (C.)	"	200	18	12	?	2000?
Chinese hamster						
somatic t.c. (H. & R.)	3 - 3.5	6 - 8	37	30 - 72	15 - 120	50
Rat						
somatic t.c. (J.P.)	3 - 3.5	6 - 8	37	23	6 - 60	31
Human diploid (J.P.)	3 - 3.5	6 - 8	37	15	10 - 70	29
Chicken						
somatic t.c.	1.45	7.5	37	25 - 30	25 - 145	63

Abbreviations:

C. = Callan (1972, 1973)

H. & R. = Huberman & Riggs (1968)

J.P. = Dr. Jean Priest (unpublished)

replication of DNA as observed in chick somatic cells and other eukaryotes and prokaryotes must be a consequence of replication progressing "spatially" in one direction while progressing "chemically" in the opposite direction as in Fig. 11 (a). Fig. 11 (b) proposes that DNA replication must first occur along complementary strands in a direction away from the initiation site. The double stranded parent DNA is then "opened" and replication proceeds discontinuously again in the 5' to 3' direction, but towards the initiation site along both complementary strands.

(iv) The number of initiation sites varies during development

Regardless of the matter of bidirectional replication, it is now known that the number of initiation sites is not fixed within one organism but may vary from tissue to tissue in relationship to S-phase (Callan, 1972). The S-phase diversity, which may occur within one organism during development, has been found in Triturus not to be accommodated by gross diversity in replication rate but by striking differences in the number of initiation sites operative for replication. This means that there is no necessary fixed direction in which a particular stretch of DNA must replicate. DNA that will replicate from right to left in a somatic cell must, in something like half the cases, be replicated from left to right during embryogenesis in Triturus.

Callan's results concerning the variation in initiation intervals found in Triturus are summarised in Table 3. The S-phase during embryogenesis has been found in many organisms to be very short compared with the S-phase of somatic cells and even more notably short when compared with the long drawn out S-phase which may be typical of pre-meiotic stages (Monesi, 1962; Callan & Taylor, 1968). Thus, Callan found that the replication units in the neurula of Triturus were extremely short (mean initiation

interval equals 43 μm) while replication units from pre-meiotic spermatocytes were very much longer (probably over 2000 μm) than those found in somatic cells (some hundreds of microns). Many initiation sites that have operated during embryogenesis will be inactive in the somatic cell, and even more will be inactive at pre-meiotic S.

It is also known that the S-phase may vary between different tissues at the same developmental stage of an organism. For example, in mouse, the S-phase in most tissues both *in vivo* and *in vitro* is between 6 and 7 hr (Killander & Zetterberg, 1965; Stanners & Till, 1960), but in certain tissues S may be much longer, for example, in the endothelial cells of the fore-stomach, it has been found to be 13.5 hr (Wolfsberg, 1964). In man the S-phase again averages 6 to 8 hr but has been found to be between 13 and 14 hr in erythropoietic and granulopoietic cells, (Stryckmans *et al.*, 1966). It seems reasonable to assume that this variation may also be accompanied by a similar variation in the number of active initiation sites.

(v) Staggering of initiations in time within a tandem sequence

Coupled with the conclusion that initiation intervals are not fixed either in development or, presumably, in different tissues, must be taken the observation that initiations of replication units within a tandem series are not synchronous but may be substantially staggered in time.

(vi) Sequential replication in S

It has been known for some years that whole chromosomes or sections of whole chromosomes are replicated during defined portions of the S-phase. The work of Taylor (1960) and Hsu (1964) on Chinese hamster cells in culture demonstrated that the Y chromosome and the long arm of the

X-chromosome were replicated only at the end of the S-phase. From work summarised by Lima-de-Faria & Jaworska (1968) it is clear that with few exceptions the heterochromatic regions of chromosomes replicate their DNA late in S-phase, and that this late replication is correlated with the heterochromatic or condensed nature of the DNA in interphase nuclei. This approach has now been extended to determine whether specific DNA sequences are replicated at a fixed time in S. Results indicate that this is the case, for example, ribosomal RNA cistrons in Chinese hamster cells replicate early in S (Amaldi et al., 1969) whereas mouse satellite DNA replicates late in S (Tobia, et al., 1970; Pardue & Gall, 1970). The time in S during which the heteropycnotic X-chromosome in female mammals is replicated has been shown to be related to its genetic expression and in some cases directly to its action on cell differentiation (Taylor, 1969). Genetic inactivation of the X-chromosome has been shown here to be associated with late replication in the S-phase; the late replication pattern first appears in some cells of embryos during gastrulation. The late replication of the X-chromosome in the male grasshopper has been shown to coincide with the differentiation of gonial cells into spermatocytes (Nicklas & Jaqua, 1965).

The staggering in the times of initiations that occurs along a stretch of replicating DNA may be a reflection of the sequential replication of specific DNA sequences, or regions as outlined above. Callan (1972) found that the temporal order of initiations did indeed vary during development. In the short S-phase in Triturus neurulae a greater degree of synchrony in initiations was observed when compared with somatic cells. Whether the variation of the temporal order of initiations is solely a consequence of S-phase duration, or whether it is linked directly to the replication of specific sequences at specific times in S, and with cell differentiation, remains unresolved; but the latter in my opinion seems more likely.

(vii) Question of control for (iv) (v) and (vi) above

Two questions may now be asked. First, how is the selection of operative initiation sites regulated; and secondly, is the same method of regulation involved in determining the time sequence of initiations?

The replicon hypothesis put forward by Jacob, et al. (1963) for bacteria, postulated two elements involved in the initiation of chromosomal replication: (1) a structure on the DNA, termed a replicator, and (2) a diffusible factor (or factors) - called the initiator - which acts on the replicator to initiate replication. It is only in eukaryotic DNA, where there are many initiation sites, that the problem of selective activation of the "replicators" or initiation sites occurs. Several models may be put forward to illuminate this problem.

Since the availability of desoxynucleotides is required for DNA synthesis throughout S, (Gurdon, et al., 1969; Nordenskjöld et al., 1970), a control on the activation of initiation sites exerted by the amount of desoxynucleotides available at any one time would seem improbable. Similarly DNA polymerase is present throughout the cell cycle (Gold & Helleiner, 1964), so even if DNA polymerase could be shown to be capable of interacting with double stranded DNA, a temporal control by the synthesis of this enzyme during S, would also seem unlikely. It seems most probable, therefore, that other initiator enzymes are responsible for the initiation of DNA synthesis at initiation sites.

Control of initiation might be effected by: (1) limitation of the number of initiator enzyme molecules produced: (2) diverse initiator base sequences and control by production of specific and complementary initiator enzymes: (3) control of mediator factors such as (a) the requirement of

protein synthesis to permit activation of initiation sites or (b) attachment of specific initiation sites to the nuclear membrane to permit their activation: (4) the marking of initiator base sequences from activation by, for example, specific variations in packing of the DNA/histone complex within chromosomes.

The first method of control assumes a single initiator base sequence as common to all initiation sites. However, if control depended solely upon the amount of initiator enzyme produced, activation of particular initiation sites during S would be random. Although this model could explain variations in number of active initiation sites between various tissues of the same organism, it does not explain the ordered replication of particular regions of the DNA at corresponding periods of S in successive S-phases.

To allow for variations in the number of active initiation sites between tissues, the second model requires not only a prior selection of which particular initiator enzymes are produced within the cell at any one time, but also a complex range of both enzymes and initiation sequences. The selective replication of regions of the DNA in the absence of replication of the rest could be taken as circumstantial evidence for the existence of diverse initiation sequences, though models (3) and (4) might serve as well. Examples of selective replication are the late synthesis of 0.3% of the genome in the pre-meiotic division cycle (Stern & Hotta, 1969) and the amplification of certain bands into DNA puffs in Sciara and Rhynchosciara. Any temporal control of initiations within the cell would require, according to this model, a further temporal control of the production of particular initiator enzymes.

As early as 1962, Mueller, *et al.*, proposed a model that protein synthesis is necessary for the activation of sites from which DNA synthesis is initiated in mammals. Experiments on cells synchronized with FUdR or amethopterin (Mueller *et al.*, 1962; Taylor, 1965) demonstrated that the amount of DNA synthesized following removal of these inhibitors (i.e. *early* in S)was reduced if protein synthesis was concomittantly inhibited, but not in late S. Other workers, however, produced conflicting results demonstrating the continued dependence of DNA synthesis on protein synthesis *throughout* the S-phase (Young 1966; Brega, *et al.*, 1960; Weiss, 1969). If protein synthesis is required for the activation of initiation sites, as postulated by Mueller *et al.*, then this requirement must presumably extend almost throughout an S-phase because initiations are thought to occur at various times during S for many cells that have been studied. This is evident because staggering of starts is observed in tandem sequences of replication units in many cells, including those of the chicken, and also because continuous labelling studies on synchronized cells have shown that replication of specific regions of the genome may be initiated late in S (see p. 60). Recent evidence from work carried out by Hori & Lark (1973) and Ensminger & Tamm (1970) suggests that protein synthesis is indeed involved in the activation of replication units. Hori & Lark claim that the addition of puromycin prevents initiation of those units which have not yet begun. As Watson (1971) has maintained it may well be impossible to judge the role of protein synthesis in the activation of initiation sites when all metabolism in the cell must necessarily be affected by inhibition of protein synthesis, including especially the synthesis of histones.

Whether protein synthesis is needed as a mediator for activation of initiation sites or not, the initial question still remains open i.e.

what is the method of control for variations in the number of operative initiation sites and the temporal control of specific initiations? Control according to model 3a would just be shifted from the production of initiator enzymes to the selective activation of specific initiation sites by some effect produced by related and concomitant protein synthesis occurring in the cytoplasm.

A similar "mediator role" has been proposed for the nuclear membrane (Comings & Kakefuda, 1968; Comings & Okada, 1973). They postulate that initiation of DNA synthesis is only possible when initiation sites are activated by contact with the nuclear membrane. This model requires only a single base sequence common to all initiation sites, as also do models, 1, 3a and 3b; but it also requires a controlled and selective change in the macromolecular arrangement of the DNA within the nucleus during S such that particular initiation sites are brought in contact with the nuclear membrane at particular times. A variable macromolecular arrangement of the DNA between cells could then account for the inactivation of certain initiation sites in particular tissues.

The main objection to this model comes from those experiments which demonstrate that contact with the nuclear membrane is not essential for DNA replication (Huberman, et al., 1973). These authors also investigated why certain experiments carried out by previous authors had been misinterpreted to suggest an association of nascent DNA with the nuclear membrane. They demonstrated that two principal sources of error had been excessively long pulses of ^{3}H-TdR and the methods of cell synchronization used. Peripheral labelling in the nucleus did indeed occur in late S in Chinese hamster cells as observed by Comings & Okada (1973). Huberman et al. found, however, that this was due to replication of heterochromatin near

the membrane, not replication in association with the membrane. Finally, their pulse-chase experiments showed that DNA is stable in position in the nucleus in Chinese hamster cells during replication. If DNA is synthesized at the nuclear membrane then the DNA should move towards the membrane for replication and away from the membrane afterwards. Another reason for doubting the role of the nuclear membrane in the activation of initiation sites comes from observations on polytene chromosomes. Places where DNA replication is occurring may be directly observed by autoradiography in polytene chromosomes; such regions are often, indeed generally, not in contact with the nuclear membrane.

Lastly, Callan (1972) proposed that control may depend on specific variations in the packing of DNA within chromosomes occurring in different tissues of an organism. Where the DNA is densely packed a smaller proportion of initiation sites would be exposed to an initiator enzyme than where the DNA is more diffusely packed. Callan supports this hypothesis with the claim that at the beginning of meiosis the overwhelming majority of the DNA is densely packed within chromomeres, and making a very rough approximation, the number of chromomeres present in Triturus lampbrush chromosomes (Mancino, 1963) is similar to the number of replication units at pre-meiotic S. Should this calculation prove to be correct it would support the idea that the chromomere, when present, is a unit of replication as suggested by Keyl & Pelling (1963) for polytene chromosomes, and that it is the macromolecular arrangement of the DNA at specific sites that controls the initiation of replication units. For this method of control to regulate a temporal sequence of initiations, a continual change in the macromolecular configuration of the DNA would have to occur during S. It may be mentioned here that 'packing' of the DNA may indeed affect the time of replication

during S of parts of the genome. For example, 'heterochromatin', generally defined as densely packed DNA in interphase nuclei, is usually absent during early embryonic stages and only appears later in development, (Lima-de-Faria & Jaworska, 1968). Correlated with this change is an alteration in the pattern of chromosome replication. Chromosomes or parts of chromosomes found as euchromatin and which replicate early in the S-phase in embryonic life, may become late replicating as they condense to form heterochromatin in the course of organogenesis (Lima-de-Faria, 1961; Utakoji & Hsu, 1965; Lima-de-Faria & Jaworska, 1968).

All models except Model 2 assume one common initiator base sequence and one type of initiator enzyme to be present within a cell. Model 1 does not explain how the selective replication of regions of the DNA could occur throughout S. Model 2 requires temporal control to be exerted by a selective and temporally controlled production of specific initiator enzymes. Model 3a suggests that controlled replication of DNA during S occurs through the selective activation of initiation sites through the products of protein synthesis. Models 3b and 4 require temporal control to be a matter of changes in the macromolecular configuration of the DNA during S. Control of the number of operative initiation sites within a cell is limited by the amount of initiator enzyme(s) produced (Models 1 and 2) the activity of mediator factors (Models 3a and 3b) or the macromolecular arrangement of the DNA (Model 4). Applying Occam's razor to the above models, that system which seems least complex in its demands on the cell would in my opinion be that outlined as Model 4.

(viii) Comparison of initiation intervals in eukaryotes and their relation to C-value

The initiation intervals in chick somatic cells range from 25 to 145 μm, with a mean of 63 μm (Fig. 7). Huberman & Riggs (1968) find most initiation intervals in Chinese hamster cells to lie within the range 15 to 120 μm, and I have computed the mean interval length from the lower histogram in their Fig. 6 to be about 50 μm. J.H. Priest (personal communication) using similar DNA fibre autoradiographic techniques has found in both rat cells in established, and in human diploid cells in culture primary culture an initiation interval range of about 10 to 70 μm with a mean of some 30 μm i.e. about half the mean distance estimated for chicken cells. The distances between initiation sites in mammalian cells and in the chicken are compared in Table 3.

On beginning this work it was anticipated that according to the unineme model for chromosome structure, the lower C-value of the chicken (1.45 μμg DNA per haploid genome: Atkin, et al., 1965) would be reflected in shorter replication units when compared with mammalian DNA at 37°C. Mammals all have a C-value between 3 and 3.5 μμg DNA per haploid genome (see review by Callan, 1972). This C-value is a little more than twice that of the chicken. The unineme model proposes that the chromatid prior to replication contains only one DNA double helix. This proposal sprang from the demonstration by Taylor, Woods & Hughes in 1957, that DNA replicates semi-conservatively in eukaryotic chromosomes. Since then much more evidence from diverse sources has accumulated in support of uninemy (Gall, 1963; Miller, 1965; Taylor, 1968; Brewen & Peacock, 1969; Laird, 1971, and Kavenoff & Zimm, 1973). The comparative study of units engaged in replication in the chromosomes of somatic cells from Xenopus

(C-value, 3.1 µµg) and Triturus (C-value 29 µµg) by Callan (1972) provides incidental support for the uninemists' claim that organisms with high C-values have longer genomes than organisms with low C-values, and not a greater number of DNA double helices in their chromatids.

The mean initiation interval in chicken somatic cells, however, is as much as twice as long as that reported for some mammalian cells and therefore demonstrates that the lower C-value of the chicken is not reflected in shorter replication units. This finding should now occasion no surprise as it was later established in the work by Callan (1972) on Triturus that neither the size of replication units nor the temporal order of their initiations were invariant; both could vary from tissue to tissue in relation to the duration of S-phase. Any relationship that might exist between C-value and size of replication units might therefore be coincidental.

(ix) Replication units and S-phase duration

The S-phase in chicken somatic cells in culture is 7.5 hr (Fig. 1). The S-phase in mammalian cells is estimated by the majority of authors to be 6 to 8 hr, (Reviewed by Cleaver, 1967). Just under half the amount of DNA is therefore replicated in a chicken cell within the same period of time. Whether this lower replication rate of overall synthesis in the chicken is accomplished by a decrease in replication rate at each growing fork is investigated in Chapter 3. However, it is assumed at this stage that staggering in the times of initiations, and longer initiation intervals, play an important role.

If the replication rate and the degree of staggering in the times of initiations is similar for mammalian and chicken cells, the DNA of

the chicken genome could be replicated within the same amount of time as that required for replication of the mammalian genome, if the mean distance between initiation sites in the chicken genome were twice that found in the mammalian genome. This is indeed the case when the mean figure for the chicken genome (63 μm) is compared, for example, to the mean figure for Rat cells (31 μm) and Human diploid cells (29 μm), according to J. Priest's results.

However, from a comparison of published DNA fibre autoradiographs from mammalian cells e.g. (Huberman & Tsai, 1973) with those from chick somatic cells, it appears that the amount of staggering of starts is more noticeable in the chick somatic cells. Tandem arrays of replication units from chicken cells are rarely observed with more than four or five replication units, whereas in mammalian cells and also in amphibian cells (Callan, 1973) much longer sequences are observed. It is deduced that this is not a result of more shear breakage of DNA from the chicken, because in Chapter 3, plates 57 to 65, many labelled tracks well over 300 μm in length may be observed after 120 min of labelling. Flanking the short labelled sequences in the chicken must be long unlabelled stretches of DNA. These unlabelled lengths might of course be a consequence of long initiation intervals; they could however also be a manifestation of a high degree of staggering in initiations.

In Chapter 3 I calculate that if DNA replication forks in the chicken genome progress at the rate estimated and the genome in its entirety takes 7.5 hr to complete replication, then longer initiation intervals than those recorded in Fig. 7 must occur and/or there must be much greater staggering of starts than is immediately apparent in the autoradiographs.

This point can be made as follows. After short periods of labelling, for example 30 min plus 30 min chase, the very fact that clusters of tandem labelled sections can be easily recognized means that within such clusters there can be relatively little staggering of times of initiations. However the clusters are themselves short, and there is no reason to suppose that they are short because of shear breakage. Therefore one must assume that regions of DNA adjacent to the labelled clusters are replicated at times significantly before, or after, the periods of labelling, i.e. that there is significant staggering of starts between, but not within, the clusters or that on either side of such a cluster neighbouring origins are a great distance away; this in my opinion is less likely.

(x) Do neighbouring replication units have similar lengths and similar initiation times i.e. are there "clusters"?

Two separate questions have been asked: (1) are neighbouring initiation intervals similar in length, (2) do neighbouring replication units have similar times of initiation? Both these questions form part of a general hypothesis: are there or are there not "clusters" of DNA consisting of, for example, 3, 4 or 5 replication units which have similar unit lengths and similar initiation times, and therefore, is the variation that is observed between the sizes of neighbouring replication units and their times of initiation really a feature of borders between neighbouring clusters, clusters which can therefore differ considerably from each other. If such clusters do occur, are they a reflection of the type of DNA being replicated, and is the variation in the replication unit size and time of initiation between clusters a reflection of variation in the type of DNA?

This proposal was put forward on account of the following observations. First, tandem arrays of labelled sections of similar size with apparently similar initiation intervals were sometimes observed immediately

adjacent to other tandem arrays, differing in both respects from the former. Secondly, similarities in labelled lengths and separation distances are often apparent within groups though these features may differ between groups in the same preparation. Because the groups being compared may not be in linear relation to one another no conclusions can be drawn here regarding the relative times of initiation between groups; however one can infer that within groups initiation intervals tend to be similar and that initiations tend to be synchronous. Thirdly, as noted on p. 70 estimates for the replication rate at a growing fork and the length of S suggest that much staggering of starts must occur. If clusters of replication units are observed to be replicating near synchronously, staggering in the time of initiations must occur between clusters.

Table 2 and Fig. 9 show that there is a tendency for like initiation intervals to occur alongside one another. This suggests that clusters of replication units of similar size do occur along a length of replicating DNA, neighbouring clusters differing from each other in this respect.

Fig. 10 tests the proposal that neighbouring replication units have similar initiation times. The tendency for points to group near the mid-line and not to be distributed randomly, indicates that in some cases this tendency does occur. If the hypothesised clusters consisted of only a few units, for example, 3, 4 or 5, the tendency for these 3, 4 or 5 units to have similar lengths and similar initiation times would not be obvious unless a great many replication units were measured. For this reason, the recording of only slight relationships in both Fig. 9 and Fig. 10, may be explained.

If members of a tandem series of replication units forming a cluster have similar initiation intervals and similar times of initiation,

the variation between clusters presumably reflects some regional and specific properties within the genome which clearly would repay further study. The sequential replication in S of various known regions of the genome in certain cells hav been discussed on p. 59 to 60 . It seems likely that the replication of these regions may be distinctive in having specific patterns of replication units. In the final analysis one would like to be able to make DNA fibre autoradiographs from known fractionated components of the genome such as, for example, nucleolar organizer DNA, so as to examine possible relationships between transcriptional and replication units.

This approach could relate the number of base pairs controlled by a replication unit with the number of base pairs determined for certain transcriptional units, for example, for ribosomal RNA cistrons (Pardue & Gall, 1970). If the average cistron is assumed to be 1000 base pairs and the distance separating base pairs is 3.4 Å (Watson & Crick, 1953) then in the somatic cells of the chicken, the mean replication unit length, 63 µm, would have under its control about 180 cistrons or 180,000 base pairs.

If neighbouring replication units tend to be of similar size and replicate during similar periods of S then it is likely that the control of initiations as discussed on p. 61 to 66 might be at the level of the cluster of tandem replication units and not at a single unit as described in Models 1 to 4.

The clustering of similarly sized replication units with similar initiation intervals differing from other clusters has recently been observed by Deaven (unpublished) in DNA fibre autoradiographs from Chinese hamster cells.

Amaldi et al. (1973), however, have presented evidence inconsistent with the proposal that neighbouring replication units tend to replicate during similar periods of S. These authors studied the reproducibility of initiation sites in two successive cell cycles in Chinese hamster cells using various techniques including DNA fibre autoradiography. Cells were labelled with ^{3}H-TdR at the beginning of two successive S-phases. Their results suggest that the position of initiation sites are reproducible from one S-phase to the next, but the time order of initiations is not. Very few examples were found to substantiate the claim, however.

The greater synchrony in the times of initiations and the shorter replication units lengths in Triturus embryos as compared to somatic cells noted by Callan (1972) also suggests that neither the timing of initiations nor the position of initiation sites are invariant.

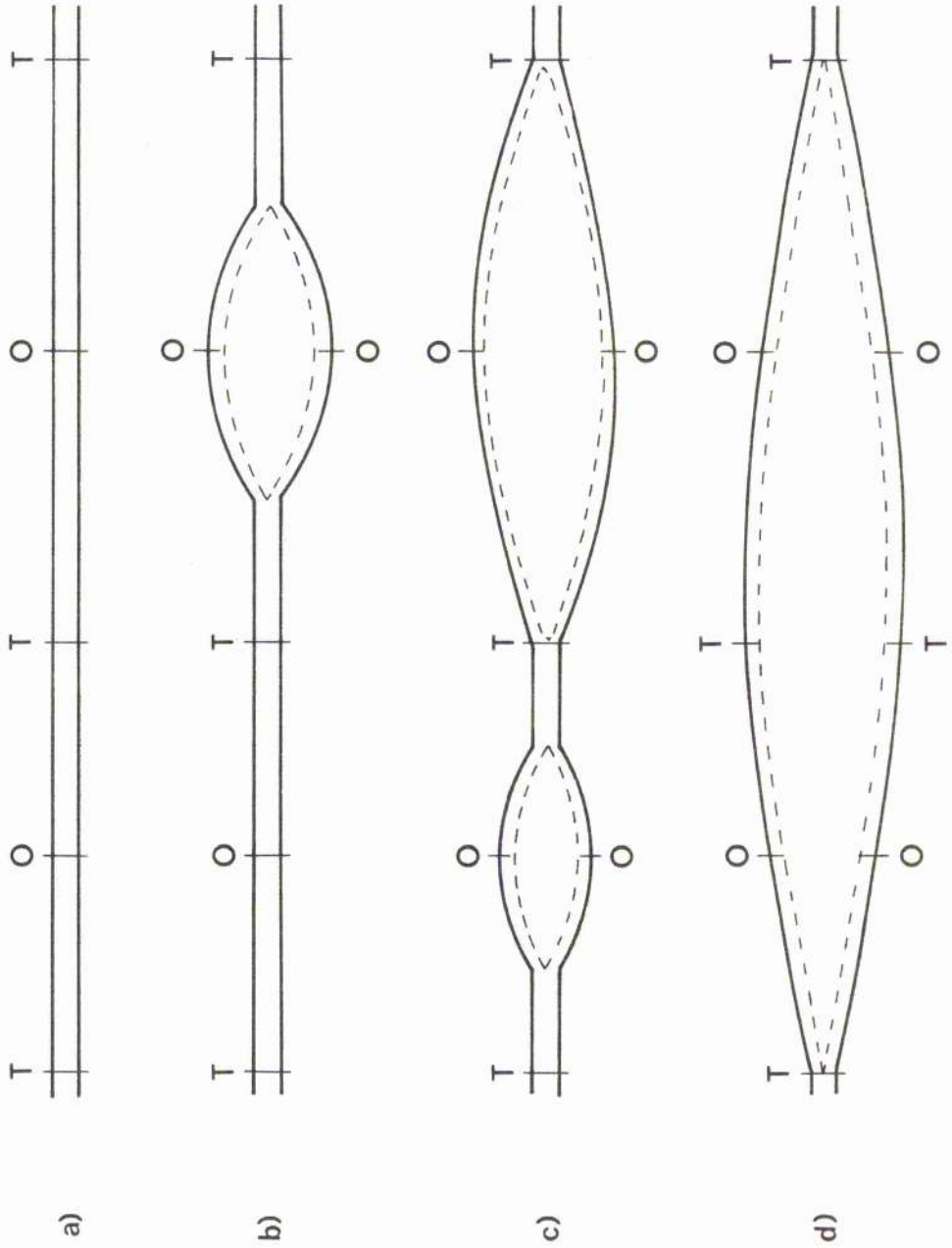
If clusters do exist, perhaps changes in the timing of initiations only occur between clusters in successive S-phases. Where synchrony occurs any sequential replication through S must be absent. Reductions in the sizes of replication units may occur regularly i.e. the size of the replication unit is halved etc., so that the similarity between the length of neighbouring replication units within a cluster need not be lost.

(xi) Termini

When Huberman & Riggs proposed their model for the replication of DNA in the chromosomes of eukaryotes (redrawn in Fig. 12) they suggested, though not with great insistence, that replication units have fixed termini. Their model also proposed that these termini might be positioned at equal distances on both sides of an initiation point.

Fig. 12. Copied from Huberman & Riggs (1968) and is their Fig. 7. It presents the bidirectional model for DNA replication. Each pair of horizontal lines represents a portion of a double helical DNA molecule; a continuous line represents a parental polynucleotide chain, an interrupted line represents a newly-synthesized chain. The short vertical lines represent origins (O), i.e. initiation points for replication, and termini (T). The four diagrams represent different stages in the replication of two adjacent replication units: (a) prior to replication (b) replication started in right-hand unit; (c) replication started in left-hand unit; (d) replication completed in both units, and sister double helices separated at the common terminus.

Fig 12



More recently, evidence for fixed termini in phage Y has been noted by Ratner & Kulitchkov (quoted by Huberman & Tsai, 1973) and in the evidence of Schnös & Inman (1970). Schnös & Inman point out that, even in cases where the rate of growth of the two forks in the Y molecule is very uneven, no fork seems to pass a particular point on the molecule located about 5.5 to 7.0 μm from the left end. This point according to Ratner & Kulitchkov may be a terminus. If the same model holds for eukaryotes the simplest situation might be to suppose that termini are fixed at equal distances along a length of DNA, regardless of the distribution of initiation sites. In Fig. 13, Models (i) and (ii), grain patterns are illustrated such as one would expect to find in DNA autoradiographs from pulse-chased cells if replication proceeded according to the above models i.e. (i) termini occurring at equal distances from an origin, or (ii) termini occurring at equal distances along the DNA chain regardless of the positions of origins. All the models in Fig. 13 assume a similar rate of progression for both growing forks produced from a shared initiation site and rely on the established data that some staggering in the times of initiations and some variation in initiation intervals may occur within a tandem sequence of replication units.

It is concluded from the present work that neither models (i) nor (ii) are satisfactory. If termini do occur, both models predict the finding, in pulse-chase experiments, of a percentage of abruptly terminating, heavily labelled tracks where replication of a unit had reached its terminus (T) during the 30 min period of labelling. Where such a heavily labelled section is flanked at its abruptly terminating end by a neighbouring heavily albelled section carrying a facing tail, the abruptly terminating end of the first section may be considered to represent the

Fig. 13. Tandem sequences of heavily-labelled units and tails which might be observed in DNA fibre autoradiographs from chick somatic cells pulse labelled for 30 min and chased for 30 min according to models of DNA replication which either include or exclude termini.

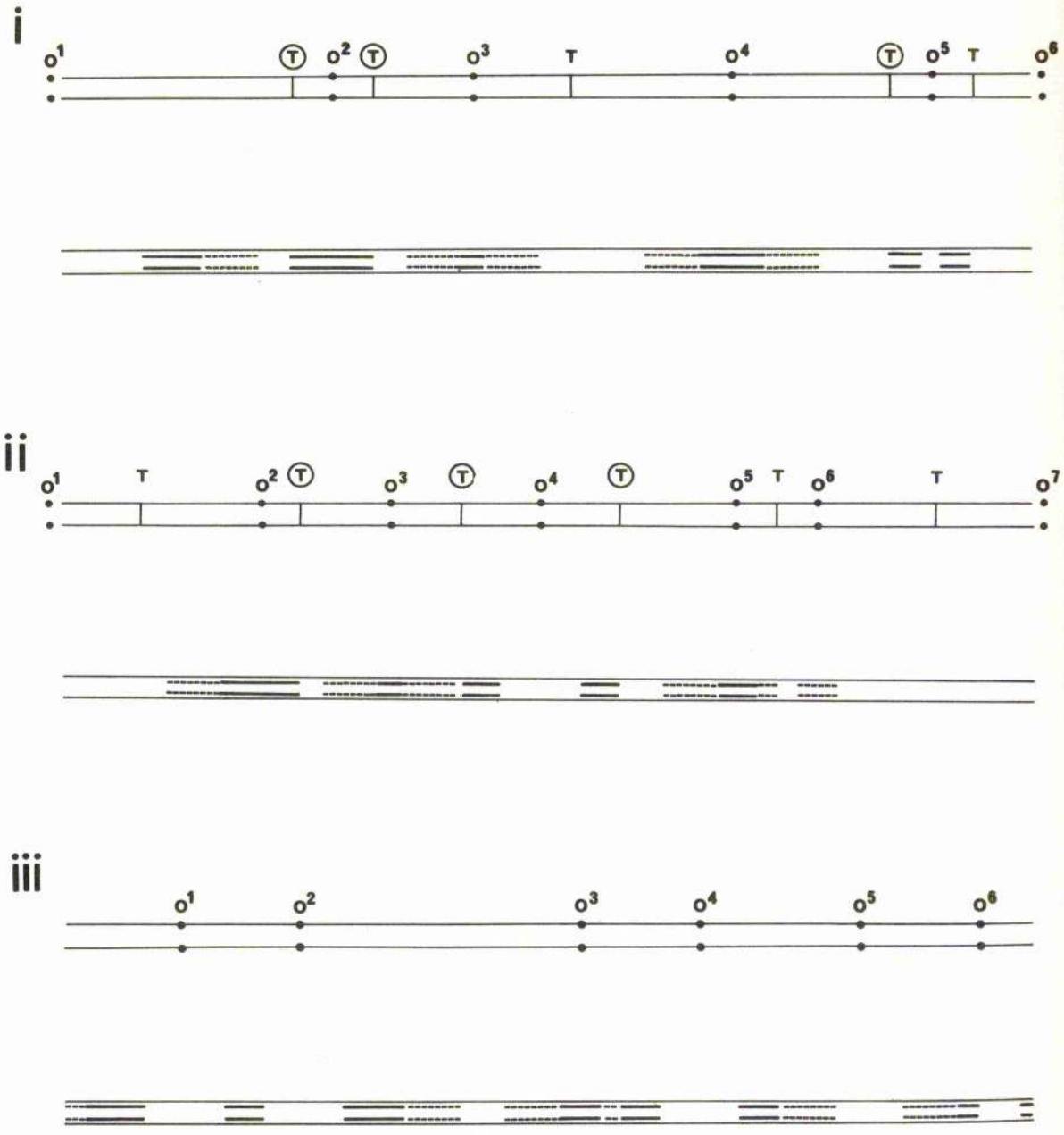
(i) Termini occurring at equal distances about an origin,

(ii) Termini occurring at equal distances along the DNA chain regardless of the positions of origins.

(iii) No defined termini. Replication is terminated when converging growing points meet. O, represents an initiation site or origin, T, represents a terminus, (T) represents a terminus which could be inferred from the autoradiograph, a thin continuous line represents a portion of a parent polynucleotide chain, a heavy solid line represents DNA synthesized during the first 30 min pulse and an interrupted heavy line represents DNA synthesized during the 30 min chase.

Abruptly terminating units which reached termini (T), during the 30 min pulse and face converging tails are shown in (i) proceeding from origins O^2 and O^5 (ii) proceeding from origins O^2 and O^4. The appearance of labelled sections which would establish that there are no defined equi-distant termini on either side of origins are illustrated in (iii) about origins O^1, O^2, O^3 and O^4.

Fig 13


i
o1
T
o2
T
o3
T
o4
T
o5
T
o6
ii
o1
T
o2
T
o3
T
o4
T
o5
T
o6
T
o7
iii
o1
o2
o3
o4
o5
o6

position of the terminus (T). Model (i) predicts that the heavily labelled sections will be abruptly terminating at both ends; model (ii) predicts the occurrence of heavily labelled sections abruptly terminating at both ends or at one end only, the other end carrying a tail. The present studies, however, reveal few such tandem series. Huberman & Riggs also found that in all of the labelled sections they observed only 3% were abruptly terminating and flanked by neighbouring replication units carrying facing tails.

Model (ii) not only predicts the occurrence of internal abruptly terminating tracks but also requires all finished replication units lengths to be the same. This is obviously not the case. For this reason also, fixed termini could not be equidistant along a length of DNA.

How then is DNA synthesis terminated at the end of each replication unit? The method which seems most likely is illustrated in Fig. 13, Model (iii): replication terminates when converging growing forks from neighbouring replication units meet. This is supported by the fact that in pulse-chase experiments I have often encountered tandem series such as shown in Plates 16 and 17 and illustrated in Model (iii) in the units arising from origins O^1 and O^2, where a middle, abruptly terminating track is flanked on both sides by labelled sections each showing a well defined tail proceeding outwards, but with an abrupt ending towards the inside. Evidently, as in Plates 16 and 17, origins lie at the mid-points of the gaps between labelled tracks, as illustrated in Model (iii), the middle track represents the outcome of replication to fusion which occurred during the pulse, whereas the outer tracks represent replication proceeding from the same initiation points and continuing during pulse and chase until harvesting of the cells. Such tracks establish without

ambiguity that replication from an origin proceeds bidirectionally without regard to symmetrically disposed termini about an origin (Model i).

If the termination of a replication unit simply depends on the meeting of converging growing forks, then it follows that neighbouring units must replicate at least in part within overlapping time periods. If this is the case, and it seems reasonable, then the "control" of initiation site activation discussed on p. 61 to p. 66 becomes in essence a matter of timing. Neighbouring and operative initiation sites, if they are greatly staggered in their times of activation must necessarily be separated by relatively long distances, whereas neighbouring initiation sites which activate synchronously can be close together. A potential initiation site then becomes inoperative if it is not activated before its neighbouring replication units at either side fuse.

The relation between a distinctive pattern of replication and the type of DNA has been suggested on p. 70 . It has also been proposed that staggering of the times of initiations through S may result in the sequential replication of certain parts of the genome (p. 60). If neighbouring replication units must replicate in overlapping time periods then perhaps a small percentage of termini (equivalent to the 3% observed by Huberman & Riggs) do exist to separate groups of replication units that do not replicate in overlapping time periods, so allowing sequential replication in S.

On the other hand parts of the genome or clusters of replication units which replicate in diverse times in S may be separated by very long initiation intervals. In this case no termini would be required.

CHAPTER 3

PULSE-LABELLED CELLS

INTRODUCTION

DNA fibre autoradiographs were prepared from chick somatic cells, pre-treated with FUdR and pulse-labelled for periods of increasing duration. The rate of DNA replication at a growing fork cannot be determined from examination of chased material because tails lack precise limits and therefore cannot be measured precisely. Replication rate must therefore be estimated from preparations derived from cells not subjected to a chase, bearing in mind the problems of interpretation exposed by the chased preparations outlined in Chapter 2. Replication rate is expressed as the rate of increase in length of a bidirectional replication unit. Where the replication rate of a single growing fork from a shared initiation site is discussed, the term one-way-replication rate is used.

METHOD

The methods for labelling the cells and preparing DNA fibre autoradiographs are outlined in the General Account of Materials and Methods. Cells in separate flasks were labelled with ^{3}H-TdR for 15-, 30-, 60-, or 120 min. The specific activities of the ^{3}H-TdR used ranged from 23 to 25 Ci/mM. After labelling the cells were immediately harvested and resuspended in concentrated sucrose solution to terminate labelling. Cells were pre-treated with FUdR and UR for 16 hr before labelling. Whole cell autoradiography showed 33% of the cells were synchronised at the beginning of S. DNA fibre autoradiographs were prepared according to the dialysis chamber method. Exposure times varied from 28 to 38 weeks.

As in Chapter 2 the length of labelled sections was measured using an eyepiece scale. Internal labelled sections from tandem arrays were measured in all cases to avoid the inclusion of broken end sections in the measurements.

RESULTS

(i) Increase in length with time

Plates 23 to 65 show the distribution of radioactivity in DNA fibre autoradiographs from FUdR-treated chick somatic cells labelled for 15 min (Plates 23 to 33), 30 min (Plates 34 to 43), 60 min (Plates 44 to 56) and 120 min (Plates 57 to 65), immediately prior to harvesting. Two main features are apparent: labelled sections occur in tandem and their lengths increase with increasing duration of labelling. Comparison of these photographs with those discussed in Chapter 2 i.e. of cells labelled for 30 min and subsequently chased for 30 min, establish that the "tails" of decreasing grain density in the latter are indeed a consequence of a chase protocol. The present labelling procedure produces heavily labelled tracks which have well-defined ends. Measurements of these lengths can therefore be made to $\pm$ 1 µm.

(ii) Sister strand separation

Several aspects of DNA replication that are more accurately determined from pulse-chase preparations can, however, also be observed in unchased preparations where there has been separation of fully replicated regions of sister double helices and where several initiation sites have been activated prior to labelling, as in Plates 39, 44, 46, 49 and 64.

The divergence of fork-like growing points in the above mentioned autoradiographs is indicative of bidirectional replication and the concurrent replication of both parent polynucleotide strands.

Unlabelled regions within such separated sister double helices must represent DNA replicated before addition of ^{3}H-TdR. Therefore, in the above mentioned plates, initiations most probably occurred in the middle of each unlabelled gap. Initiation intervals may be measured in these cases as the distance from mid-gap to mid-gap. Intervals measured from these unchased preparations lie within the range shown in Fig. 7, which derives from pulse-chased cells. For example, intervals of about 40 and 50 μm are estimated directly from Plate 39 and intervals of 50 μm and 90 μm from Plates 44 and 49 respectively. Thus, neighbouring initiation intervals, measured in this way, also show variations in length.

Both chased and unchased preparations demonstrate that the time of initiations may vary between replication units. For example, if initiation sites occur at the mid-gaps in the tandem sequence shown in Plate 49, then the difference in length between these unlabelled regions indicates that initiation of the right-hand unit must have occurred before the left-hand unit, both units initiating prior to labelling, assuming that replication rate is invariant.

Only pulse-chase experiments provide unequivocal evidence against there being fixed termini. However, tracks from unchased material showing sister strand separation as in Plates 44 and 49 are also most easily interpreted on the basis of the lack of defined termini equidistantly spaced about an origin. For example, in Plate 44, if origins are taken to lie at the middle of each unlabelled gap, then the internal labelled track represents the outcome of fusion of the neighbouring one-way replication

whilst the outermost one-way replication units have continued to replicate for a much greater distance until harvesting of the cells. This is assuming that fusions have not occurred between the outermost units and other labelled sections.

(iii) Interpretation of a tandem array

Fig. 14 shows an interpretation of the tandem sequence of replication units in Plate 39, (from cells labelled for 30 min) showing sister strand separation. Origins are inferred at positions O_1, O_2, O_3 and O_4. (1) represents the probable arrangement of replicated lengths before label was available. Initiation has occurred at origins O_1, O_2 and O_3. (2) represents the supposed arrangement of labelled tracks after 15 min of labelling. Initiation has now occurred at origin O_4. (3) represents the sequence of labelled tracks at the end of the labelling period i.e. Plate 39 redrawn. Fusion has now occurred between all converging one-way sections and the fully replicated sister strands have separated.

Several uncertainties in this interpretation must be mentioned. Whilst origins probably occurred at positions O_1, O_2 and O_3, each gap might contain more than one origin and DNA synthesized from these origins may have fused prior to labelling. Again, origin O_4 may be valid, but its labelled length could also represent a one-way section from origin O_3, (providing an example for the absence of equi-distant termini about an origin) rather than being a two-way replication unit.

(iv) Calculation of replication rate/growing fork

Ideally, the labelled length of a replication unit when measured after a pulse-label of known duration should be proportional to the rate of DNA replication from two diverging growing forks. However, in these

Fig. 14. An interpretation of the pattern of replication along a portion of a DNA molecule from the arrangement of tandemly labelled sections of nascent DNA showing sister strand separation, as observed in a DNA fibre autoradiograph prepared from chick somatic cells labelled for 30 min. A thin continuous line represents a parent polynucleotide chain, a dotted line represents DNA replicated before labelling, and a heavy continuous line represents DNA replicated in the presence of label. O, represents an origin or initiation site. Redrawn from Plate 39.

(1) Pattern of replication prior to labelling.

(2) Pattern of replication that would have been observed if cells were harvested after the first 15 min of the pulse label.

(3) Pattern of replication as observed in Plate 39, after 30 min labelling.

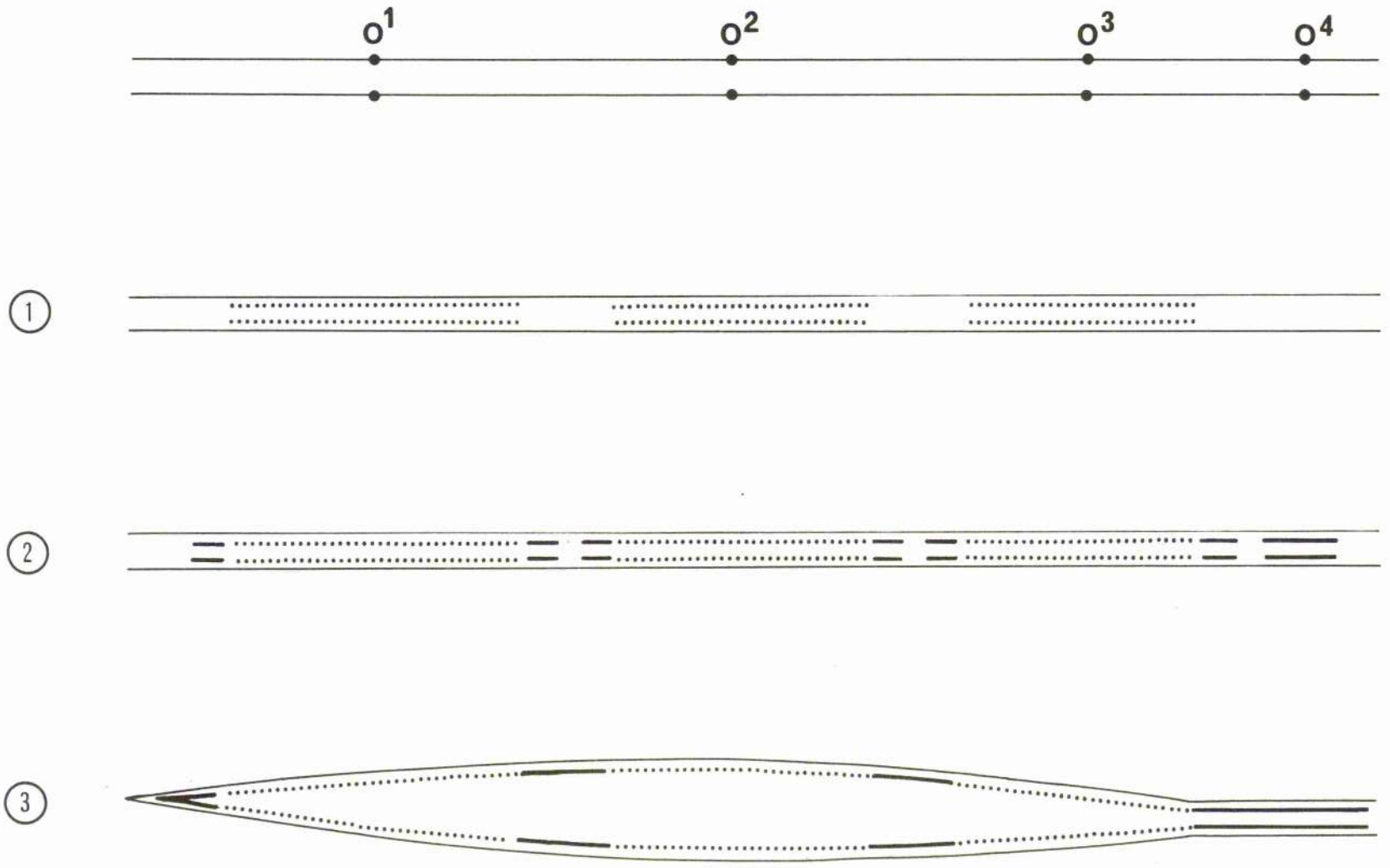


Fig 14

autoradiographs the tracks measured are necessarily an indeterminate mixed population.

(1) Some labelled sections will indeed represent replication proceeding in opposite directions from a shared origin which initiated immediately after removal of the FUdR block, and with no subsequent fusion between neighbouring replication units.

(2) However, other such sections will have fused with neighbouring labelled sections, resulting in longer labelled tracks than those in (1).

(3) Other labelled sections will also reflect two-way replication, but where initiation was delayed, thus resulting in shorter labelled tracks than those in (1).

(4) These lengths may also have fused with their neighbours, resulting in intermediate lengths depending on when fusion occurred and the labelled lengths of their neighbours.

(5) Yet other labelled sections will reflect one-way replication diverging from an already replicated region that initiated before label was available. These short labelled lengths would thus be a measure of one-way replication rate.

(6) Such labelled sections would again result in intermediate length if fusion had occurred between neighbouring replication units.

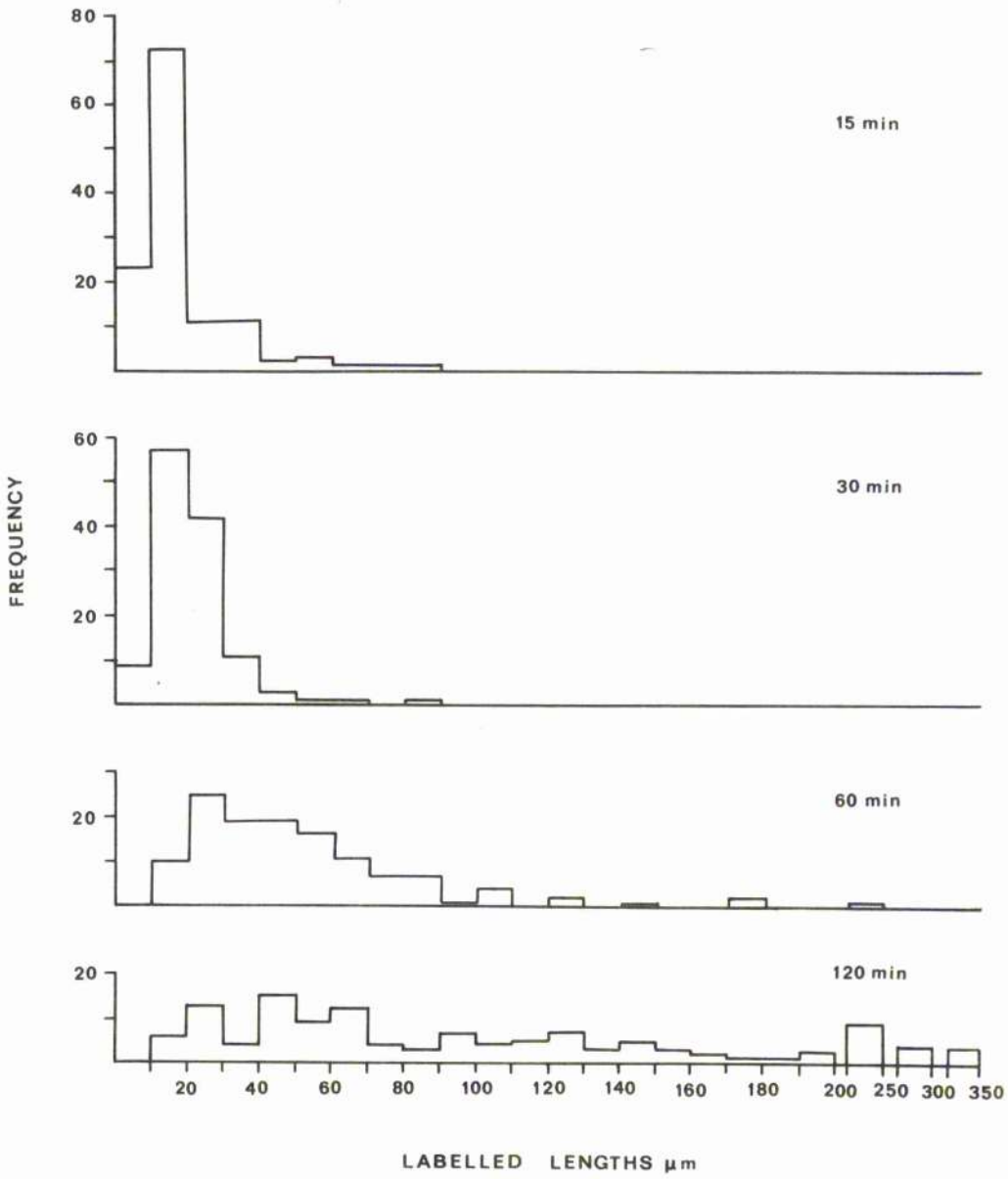
(7) Lastly, certain long labelled tracks might be caused by fusion between neighbouring replication units which initiated from nearby origins at some time during labelling.

The proportional contribution of all the above mentioned circumstances would vary inter alia with duration of labelling. Only exceptionally is it possible to recognize, with fair assurance, the provenance of a particular labelled track in unchased preparations. In practice, however, the proportional contribution of the first mentioned track lengths can be increased by the use of an FUdR block and by labelling the cells for a time for which it has been empirically established that fusion is rare. If cells are labelled immediately an FUdR block is released, those cells synchronized at the beginning of S will initiate replication and DNA replicated at this time will be labelled. The proportion of replication units that initiate immediately label is provided must be greater in semi-synchronized cells, a third synchronized at the beginning of S, than if unsynchronized cells were labelled, even allowing for many initiations to be staggered in time through an S-phase.

Fig. 15 shows histograms of the labelled track lengths measured from DNA fibre autoradiographs from cells pre-treated with FUdR for 16 hr and labelled for 15-, 30-, 60-, and 120 min. 125 measured lengths were recorded for each histogram. The great spread of lengths in the 60- and 120 min histograms is clearly the result of fusion, whereas the compact 15 and 30 min histograms are evidence that little fusion has occurred between neighbouring replicating sections which initiated within these shorter periods of labelling. The mean values of labelled lengths recorded for each histogram are 18, 23, 51, and 106 μm respectively. The very low proportion of lengths equal to twice that of the mean length of more, in the 15 and 30 min histograms, is evidence that within a labelling period of 30 min or less few fusions occur between two or more neighbouring replication units that initiated at or after the beginning of labelling. This establishes,

Fig. 15. Frequency histogram of labelled track lengths from DNA fibre autoradiographs derived from chick somatic cells in culture. The cells were treated with FUdR and labelled with ^{3}H-TdR for 15, 30, 60 and 120 min.

Fig 15


80
60
40
20
15 min
60
40
20
30 min
FREQUENCY
20
60 min
20
120 min
20 40 60 80 100 120 140 160 180 200 250 300 350
LABELLED LENGTHS µm

empirically, that the replication rate may be measured with more accuracy from autoradiographs of chick somatic cells labelled for 30 min or less.

Because FUdR pre-treatment for 16 hr only results in a third of the cells actually beginning their S-phase at the start of labelling, a large proportion of one-way sections will necessarily have been measured. For this, and other reasons previously mentioned, the mean track lengths do not give a fair measure of replication rate. In order to estimate this rate, the maximum unfused lengths recorded in a given labelling period are required. The right-hand shoulder (RHS) of the 15 and 30 min histograms give such a value for the maximum two-way lengths recorded, with a minor contribution from fusion of one-way sections that terminated when labelling ceased. Beyond each RHS is the low percentage of irregular track lengths resulting from fusion. To the left of each RHS are track lengths resulting from, (i) one-way replication units from origins that initiated before label was available i.e. cells already in S when FUdR was applied, (ii) two-way replication units where initiation was delayed until some time after label was available, (iii) the fusion of two converging one-way units, where fusion occurred early in the labelling period or (iv) replication units from extremely close initiation sites that initiated and fused during labelling. The RHS of both the 60 and 120 min histograms are 'masked' since the proportional contribution of all types of fusions is overwhelming after these periods of labelling. The RHS of the 15 min histogram is between 10 and 20 μm and if one takes its mean, 15 μm, the replication rate is 30 μm/hr one-way. The RHS of the 30 min histogram is between 20 and 30 μm, giving an estimated replication rate of 25 μm/hr one-way. Although it is by no means certain that replication rate is invariant I consider a rate of some 25 to 30 μm/hr one-way to be a reasonable estimate, for it is supported by two further observations.

TABLE 4. DNA REPLICATION RATE IN MAMMALIAN AND CHICK SOMATIC CELLS

Cell Type	Rate as published	Rate one-way per hour	Reference
HeLa S3	0.5 - 0.8 μm/min	15 - 24	a
HeLa	0.5 μm/min	15 or less	b
Chinese hamster	0.7 - 1.1 μm/min	21 - 33	a
" "	30 - 72 μm/hr	30 - 72	c
" "	1 μm/min	30	d
" "	0.8 μm/min	24	e
" "	1 - 2 μm/min	30 - 60	f
Mouse	0.6 - 0.7 μm/min	18 - 21	g
" L	0.7 - 1.8 μm/min	21 - 54	a
Human Diploid	15 μm/hr	15	h
W1 - 38	0.9 μm/min	27	a
Kangaroo rat	1 μm/min	30	d
CRL (rabbit)	0.5 - 0.8 μm/min	15 - 24	a
Rat	23 μm/hr	23	h
Chick somatic	25 - 30 μm/hr	25 - 30	i

a - Painter & Schaefer (1969)
b - Cairns (1966)
c - Huberman & Riggs (1968)
d - Deaven (unpublished)
e - Lark et al. (1971)
f - Taylor (1968)
g - Hand & Tamm (1973)
h - Priest (unpublished)
i - McFarlane & Callan (1973)

First, because the mean initiation interval is 63 μm (see Fig. 7, Chapter 2) the above replication rate would result in fusions between units which initiated replication during the labelling period only becoming numerous after about 60 min of labelling and from the 60 min histogram in Fig. 15 this is evidently the case. Secondly, in the photographs in Chapter 2 of tandem labelled lengths from cells labelled for 30 min and chased for 30 min, fusion is seen to be occurring at the tails of neighbouring replication units in the majority of cases.

The replication rate per growing fork in chicken somatic cells and mammalian cells is compared in Table 4.

DISCUSSION

(i) Correlation of replication rate and initiation intervals

The estimated value for replication rate, 25 - 30 μm/hr one-way, has been shown to agree with the information presented in Fig. 7, Chapter 2, for the mean initiation interval. This correlation can, however, be taken further. If the value for rate is correct and assuming this rate to be invariant, the percentage of fusions after 30 min labelling, between neighbouring replication units that initiated when ^{3}H-TdR was added, would be expected to equal the percentage of initiation intervals below 30 μm from Fig. 7. From 151 initiation intervals recorded 5 examples (3.3%) were below 30 μm. However, as mentioned by Callan (1972) replication units proceeding from any two neighbouring initiation points which do not fuse within 30 min contribute two labelled tracks on an autoradiograph, whereas two which fuse contribute only one. Thus, the expected frequency of fusions in 30 min would be 1.6%. From 125 measurements of labelled

lengths recorded after 30 min labelling, only 3 long labelled tracks, greater than 50 μm (about twice the mean), were recorded, a frequency of 2.4%.

(ii) Correlation of replication rate and S-phase duration

If the most widely-spaced origins in chick somatic cells are some 145 μm apart (Fig. 7) and if all the replication units were to initiate at about the same time and continue replication at a constant rate of 25 to 30 μm/hr one-way then all the DNA of the chicken genome could be replicated in about 2.5 hr. The actual S-phase is, however, 3 times longer (Fig. 1 Chapter 1); how is this disparity to be explained?

The possibility that initiation intervals longer than 145 μm were not recognised has already been mentioned on p. 45 , Chapter 2, and cannot be refuted.

Another possibility is that initiation intervals may tend to be longer as the S-phase progresses; this, however, can be contested. Initiation intervals measured from tracks where sister-strand separation has occurred i.e. from regions of the genome whose replication is not confined to early S, do not differ significantly from intervals measured in early S i.e. from chased preparations where initiation occurred after relief of the FUdR block. On p. 79 , intervals were determined from unchased preparations from tracks where sister strand separation had occurred and such intervals fell within the same range as those in Fig. 7. These determinations can be made on preparations labelled up to 120 min after the release of the FUdR block and there120 min after the start of S in a third of the cells. It is also noted in Chapter 4 that initiation intervals measured from an unsynchronized population of cells also lie within the same range as those in Fig. 7.

Yet another possibility is that replication rate falls during S. Previous authors, however, have presented evidence that variations in the length of the S-phase within one organism or between related groups of animals, is not determined by variations in replication rate. For example, Callan (1972), Table 3, Chapter 2, found that the diversity in S-phase duration in Triturus somatic cells, spermatocytes and neurulae, was not accommodated by gross diversities in replication rate but by variations in the number of initiation sites. Painter & Shaefer (1969) estimated the DNA replication rate for cells of 5 mammalian species in vitro by density labelling (Table 4). The S-phase in HeLa, Chinese hamster, Mouse, L, and Human diploid cells is generally 6 to 8 hr, (reviewed by Cleaver, 1967), but the S-phase in C.B.L. rabbit brain cells is some 25 hr (Painter & Shaefer, 1969). They found that all 5 cell lines have similar two-way replication rates, 0.5 μm - 1.8 μm/min, and therefore the long drawn out S-phase in rabbit C.B.L. cells is, as in the situation noted by Callan, not accommodated by a decrease in replication rate. It seems unlikely, therefore, that replication rate changes significantly within one S-phase.

A last possibility is that the length of the S-phase in chick somatic cells may be a result of staggered initiations in time. Although those neighbouring origins which are both going to be activated in the same S-phase must initiate in overlapping time periods, staggering of such initiations does occur. The longer the interval between adjacent active initiation sites the greater the degree of staggering that can occur in the time of their initiations (See Chapter 2, p. 76). A staggering of starts of up to 2 hr can be observed in certain autoradiographs. For example in Plate 62, if, as seems most likely, the unlabelled central region locates a cluster of replication units which had not

initiated before harvesting and if the neighbouring labelled sections represent clusters of two-way replication units which initiated at the beginning of labelling (as seems probable from the length of these sections) then a staggering of starts of some 2 hr may be inferred. Staggering in the time of initiations has also been proposed to explain the long unlabelled stretches flanking short clusters of labelled sections (see p. 70 , Chapter 2 and many examples shown in Plates 23 to 65). This final proposal is in my opinion the most likely explanation for the observed discrepancy between the length of S, the mean replication unit length and the replication rate.

(iii) Amount of DNA replicating at any one time in S

A rough estimate for the amount of DNA replicating concurrently at any one time in S may be made using the calculated value of replication rate if it be assumed that the degree of staggering in the time of initiations remains constant throughout the S-phase. If the mean initiation interval is 63 μm (Fig. 7) the S-phase is about 7.5 hr (Fig. 1) and the replication rate is 25 to 30 μm/hr one-way, only about one seventh of the total DNA of the chicken genome would be replicating at any one time in S.

63 μm ÷ 25 - 30 μm/hr one-way = approx. 1 hr

but S = 7.5 hr ∴ 1 ÷ 7.5 = approx $\frac{1}{7}$.

(iv) Comparison of DNA replication rates at 37°C

A wide variation exists in figures for the rate of progress of replication forks in DNA from mammalian cells at 37°C (Table 4), but

most of this variation probably stems from the complications mentioned earlier on p. 81. In Table 4 results are converted to one-way replication rates per hour wherever it is obvious that values were gained by the said authors from a majority of bidirectional replication units. These converted rates may now be compared more successfully, although where conversions are made, an underestimate is still probable in all cases since the rates were calculated from the mean value of lengths recorded in a given labelling period. Both Painter & Shaefer (1969) and Taylor (1968) obtained values for replication rates using the density label 5-bromouracil (BrdU). Comparison of the converted replication rates shows that most determinations of the replication rate of mammalian DNA lie within the same range, about 15 to 60 μm/hr one-way and that the replication rate determined in the present study for DNA from chick somatic cells also lies within this range.

(v) DNA replication rate, C-value and S-phase duration

The duration of S is similar in chick somatic cells *in vitro*, 7.5 hr (Fig. 1) and in mammalian cells *in vitro* generally 6 - 8 hours (reviewed by Cleaver, 1967). If these estimates are correct and the C-value of the chicken is just under half the amount determined for mammalian cells (reviewed by Callan, 1972) then half the amount of DNA is replicated in the chicken at the same rate per growing fork as in mammals over the same amount of time. The lower replication rate in chick somatic cells must therefore be accommodated by either a significant reduction in the number of initiation sites or a greater degree of staggering in the time of initiations, or both.

As has been seen in Chapter 2, Table 3, the mean initiation interval in chick somatic cells is longer and may even be twice as long as the mean

initiation interval calculated for certain mammalian cells. Therefore, if chicken DNA replicates with half the number of initiation sites at the same rate per growing fork, for the same duration of time, half the amount of DNA would be replicated! If the number of initiation sites in mammalian cells is less than twice that of the chicken, as estimated by Huberman & Riggs (1968) for Chinese hamster cells, then a greater degree of staggering in the time of initiations must occur in the chicken genome as compared with the mammalian genome.

(vi) Replication rate and number of replication units

Before any estimates for the mean initiation interval were found by DNA fibre autoradiography, the mean replication unit length and the number of replication units in the entire genome were calculated for various organisms from estimates of DNA replication rate, C-value and S-phase duration. From such values Taylor (1968) estimated that replication units in the genome of Chinese hamster cells were 200 to 400 μm in length. Similarly, Cairns (1966) working with HeLa cells calculated two-way units of 300 μm. All such value, however, must underestimate the number of replication units and overestimate the mean replication unit length because one must assume in these calculations that all units initiate at the beginning of S and replicate continuously throughout S, whereas it is known that the times of initiations may be staggered. Indeed, DNA fibre autoradiographs of both HeLa and Chinese hamster cells have shown that staggering in the times of initiations does occur (Huberman & Riggs, 1968; Lark et al., 1971; Deaven, unpublished).

Callan (1972) using the above calculations and making a rough allowance for staggered starts, estimated that 4,500 two-way replication units

may be involved in replication in the pre-meiotic S in Triturus. If this number is correct, initiation sites must average 2.4 mm apart.

Assuming uninemy, the haploid genome length in the chicken is about 50 cm. A similar calculation for the chicken, but not allowing for staggered starts would be 7.5 hr x 25 to 30 μm/hr one-way, 188 - 225 μm. The number of replication units would then be about 2,200. The mean replication unit length is 63 μm, however, not some 400 μm. A more valid estimate for the number of replication units in the chicken genome would be 50 cm ÷ 63 μm, or about 8,000.

(vii) Replication rate in eukaryotes and prokaryotes

Finally, note must be taken of the markedly slow replication rate per growing fork recorded for all eukaryotic DNA, including that of the chicken, compared with the rates of replication recorded for prokaryotic cells. At 37°C replication of DNA in bacteria proceeds at a rate of about 20 - 30 μm/min, one-way, (Cairns, 1963; Bonhoeffer & Gierer, 1963; Cooper & Helmstettxer, 1969) and is complete in E. coli within 20 to 30 min (Cairns, 1963). At 37°C the replication rate in eukaryotes is between about 0.16 to 1.2 μm/min one-way (Table 4). The DNA of the chicken genome (replication rate 25 to 30 μm/hr one-way) replicates at a rate about 60 times slower than E. coli DNA and is complete in 7.5 hours. It is also of interest that the E. coli replicon calculated to be 1,100 to 1,300 μm in length with a mol. wt. of 2.5×10^9 daltons is also much longer than any recorded lengths for replication units in eukaryotes, and about twenty times longer than the mean replication unit length (63 μm) in chick somatic cells. The rate of DNA replication in polytene chromosomes also supports the above generalization. The rate of DNA replication in Rhynchosciara angelae is 0.025 μm/min at 24°C (Cordeira & Meneghini, 1973) and correcting

for the difference in temperature, they calculate that the rate would be about 0.05 μm/min at 37°C.

An explanation for the relatively fast rate of replication in prokaryotes may be the lack of associated histones in prokaryotic chromosomes. In eukaryotes histones are closely linked with the DNA in chromosomes. Double stranded DNA must unwind before replication is possible. The unwinding of histone-associated DNA is presumably more complex and may therefore be slower than the unwinding of free DNA. On the other hand, the association of histones with nascent DNA may take place during replication, as an integral step in the replication process. It follows, if this were the case, that inhibition of protein synthesis might affect the rate of DNA replication in eukaryotes but not in prokaryotes. This is found to be correct (Hanawalt, et al., 1961; Lark, 1963; Cummins & Rusch, 1966; Mueller et al., 1962; Weiss, 1969; Weintraub, 1972b). Weintraub, for example, showed that when protein synthesis in chick erythroblasts was inhibited by cycloheximide, linear DNA synthesis continued for about 45 min, but at half the control rate. Other authors (Gautschi & Kern, 1973) agree with Weintraub's work that it is the rate of chain elongation of nascent DNA that is reduced, not the number of active initiation sites.

It is proposed, therefore, that the need to incorporate proteins plays an important role in restricting the rate of DNA replication in eukaryotes.

CHAPTER 4.

SOMATIC CELLS NOT TREATED WITH FUdR

INTRODUCTION

In view of the evidence that FUdR may cause chromosome breaks (Taylor, *et al.*, 1962), may therefore conceivably be responsible for bidirectional as opposed to unidirectional replication (Amaldi, *et al.*, 1972; Lark, *et al.*, 1971; Weintraub, 1972) and may not block DNA synthesis completely (Amaldi *et al.*, 1972), DNA fibre autoradiographs were also prepared from non-synchronized, non-FUdR-treated cells. The characteristics of replication units from FUdR-treated and non-FUdR-treated cells were then compared. DNA fibre autoradiographs from non-synchronized cells also provide information concerning the pattern of replication throughout S.

METHOD

The general procedures have been outlined in the General Account of Materials and Methods. Non-synchronized chick somatic cells, untreated with FUdR, were allowed to reach log growth. Three types of labelling procedures were carried out on these cells. (i) Some were labelled according to the pulse-chase protocol outlined in Chapter 2. Growth medium containing ^{3}H-TdR (Specific Activity, 27 Ci/mMol), was applied for 30 min, removed, and replaced with non-radioactive medium for a further 30 min chase period. (ii) Others received an initial 30 min pulse of ^{3}H-TdR followed by a step-up in the specific activity of the label. Growth medium containing ^{3}H-TdR at 6.75 Ci/mMol was applied for 30 min, removed, and replaced with grwoth medium containing ^{3}H-TdR at

27 Ci/mMol for a further 30 min. (iii) other cells were labelled with ^{3}H-TdR (Specific Activity 27 Ci/mMol) for periods of increasing duration: 15, 30, 60 and 120 min.

In all experiments the labelling or chase period was terminated by harvesting the cells with trypsin and immediately resuspending them in concentrated sucrose solution, pre-cooled to 0°C.

DNA fibres were isolated with dialysis chambers and the autoradiographs were exposed for 28 weeks.

Initiation intervals and labelled lengths were measured as in previous chapters.

RESULTS

(i) Thymidylate pool

The step-up labelling procedure gave few grain density gradients distinguishable in the DNA fibre autoradiographs prepared from somatic cells untreated with FUdR. It is therefore probable that a difference in specific activity greater than 4-fold would be required to produce recognizable and consistent grain density shifts in fibre autoradiographs of this material.

Grain density shifts are apparent in DNA fibre autoradiographs prepared from non-FUdR-treated, pulse-chased cells although here too, such shifts are not pronounced, (Plates 71, 72, 76 and 77). Indistinct grain density shifts must be accounted for by dilution of the initial pulse label by the existing pool of thymidylate molecules.

That the size of the pool of thymidylate molecules is significant in cells untreated with FUdR is borne out by the fact that all labelled sections from non-FUdR-treated cells, labelled according to either of the three procedures, have lower grain densities than labelled sections in DNA fibre autoradiographs from FUdR-treated cells described in Chapters 2 and 3.

(ii) Direction of replication

Where grain density shifts are observed the arrangement supports a bidirectional model of replication or is ambiguous (See Fig. 4, Chapter 2); in no cases is it incompatible with a bidirectional model. (See for example Plates 71, 72, 76 and 77).

Unequivocal evidence for bidirectional replication is provided only by clear grain density shifts, but evidence comes also from a consideration of the labelled lengths in DNA fibre autoradiographs from unchased cells, if such cells are unsynchronized. If the replication rate of DNA established for cells pre-treated with FUdR (25 - 30 μm/hr one-way) holds also for cells untreated with FUdR, then after short periods of labelling, for example 15 or 30 min, a great majority of short labelled sections representing unfused one-way units should be observed if replication is bidirectional because short periods of labelling would be expected to reduce the frequency of labelled tracks derived from fused convergent one-way sections. Many labelled lengths should, therefore, be some 6 - 7.5μm in length after 15 min labelling and some 12 - 15 μm after 30 min labelling. Frequency distributions of labelled lengths recorded from DNA fibre autoradiographs prepared from unsynchronized cells untreated with FUdR and labelled for 15, 30, 60, and 120 min are shown

TABLE 5. Frequency distribution of initiation intervals from chick somatic cells untreated with FUdR, labelled with ^{3}H-TdR for 30 min and chased for 30 min.
i., ii., and iii represent experiments on different primary cell cultures.

i.		ii.		iii.	
initiation intervals/µm	frequency	initiation intervals/µm	frequency	initiation intervals/µm	frequency
11 - 15	0	11 - 15	0	11 - 15	0
16 - 20	0	16 - 20	2	16 - 20	0
21 - 25	1	21 - 25	1	21 - 25	1
26 - 30	1	26 - 30	2	26 - 30	2
31 - 35	3	31 - 35	2	31 - 35	2
36 - 40	11	36 - 40	3	36 - 40	4
41 - 45	5	41 - 45	3	41 - 45	1
46 - 50	6	46 - 50	3	46 - 50	3
51 - 55	9	51 - 55	2	51 - 55	1
56 - 60	5	56 - 60	0	56 - 60	1
61 - 65	3	61 - 65	1	61 - 65	0
66 - 70	3	66 - 70	3	66 - 70	2
71 - 75	2	71 - 75	0	71 - 75	0
76 - 80	0	76 - 80	1	76 - 80	0
81 - 85	0	81 - 85	0	81 - 85	1
86 - 90	0	86 - 90	1	86 - 90	1
91 - 95	0	91 - 95	0	91 - 95	0
96 - 100	0	96 - 100	0	96 - 100	2
101 - 110	0	101 - 110	0	101 - 110	1
111 - 120	0	111 - 120	1	111 - 120	1
121 - 130	1	121 - 130	0	121 - 130	0
131 - 140	0	131 - 140	0	131 - 140	1
141 - 150	0	141 - 150	0	141 - 150	1
TOTAL	50		25		25
MEAN	50		49		65

in Fig. 16. The commonest lengths from the 15 and 30 min labelling experiments are indeed those to be expected if replication rate is as established for FUdR-treated cells and if replication is bidirectional.

(iii) Initiation intervals

The distances between neighbouring initiation sites are measured in DNA fibre autoradiographs from pulse-chased cells untreated with FUdR according to the method outlined in Chapter 2. The problems that must be borne in mind when analysing such measurements are also discussed in Chapter 2. Table 5 shows the frequency distribution of initiation intervals measured from three separate experiments using three different primary chick somatic cell cultures, all cultures being untreated with FUdR. Because recognisable grain density shifts were infrequent a total of only 100 measurements could be made. The results of the three experiments are shown independently to demonstrate the natural residual variation that occurs when measuring such data.

The mean initiation interval from experiments (i), (ii), and (iii) is 50, 49, and 65 μm, respectively. The ranges in the three experiments are 23 - 126, 18 - 116 and 24 - 150 μm, respectively. The longer mean initiation interval in experiment (iii) results from the measurement of several very long initiation intervals and is not due to a change in the mean length of the more frequently observed intervals. In all three experiments the majority of initiation intervals are between about 38 and 50 μm.

The initiation intervals measured in DNA fibre autoradiographs from cells untreated with FUdR (the results from experiments (i), (ii), and (iii) combined) are compared in Table 6 with those made from

TABLE 6. Frequency distribution of intervals between initiation points for DNA replication derived from chick somatic cells treated and untreated with FUdR, labelled with ^{3}H-TdR for 30 min and chased for 30 min.

+ FUdR		- FUdR	
initiation intervals/µm	frequency	initiation intervals/µm	frequency
10 - 15	0	10 - 15	0
16 - 20	0	16 - 20	2
21 - 25	0	21 - 25	3
26 - 30	5	26 - 30	5
31 - 35	11	31 - 35	7
36 - 40	5	36 - 40	18
41 - 45	16	41 - 45	9
46 - 50	14	41 - 50	12
51 - 55	12	51 - 55	12
56 - 60	10	56 - 60	6
61 - 65	14	61 - 65	4
66 - 70	16	66 - 70	8
71 - 75	13	71 - 75	2
76 - 80	7	76 - 80	1
81 - 85	4	81 - 85	1
86 - 90	2	86 - 90	2
91 - 95	4	91 - 95	0
96 - 100	4	96 - 100	2
101 - 110	4	101 - 110	1
111 - 120	2	111 - 120	2
121 - 130	4	121 - 130	1
131 - 140	3	131 - 140	1
141 - 150	1	141 - 150	1
TOTAL	151		100
MEAN	63		54

pulse-chased cells, pre-treated with FUdR for 16 hr. 151 measurements are shown in column (1) with FUdR, whereas only 100 measurements were made to compile column (2) without FUdR. The initiation intervals in column (1) are those previously plotted as a frequency distribution in Fig. 7.

The mean initiation interval is 63 µm in FUdR-treated cells, and is 54 µm from the total measurements made from cells untreated with FUdR. The range of initiation intervals is 25 - 145 µm in the former and 18 - 150 µm in the latter. There is little difference in either the range or the mean value of initiation intervals between cells pre-treated with FUdR for 16 hr and untreated cells. The slightly lower mean value for non-FUdR-treated cells may result from misinterpretation of one-way labelled sections as two-way labelled sections, if a grain density gradient is mistakenly identified at both ends of such a one-way section. The distance between two neighbouring labelled sections, one or both of which have been misinterpreted as two-way units, would mean that a short apparent "initiation interval" would be scored. Many more one-way labelled sections will be present in preparations from non-FUdR treated cells, and therefore such misinterpretations are more likely to occur.

(iv) Labelled lengths and replication rate

Plates 66 to 85 are high-power photomicrographs showing the distribution of radioactivity in DNA fibre autoradiographs from unsynchronized, non-FUdR-treated cells, labelled for 15 min (Plates 66, 67 and 79), 30 min (Plates 68 - 70 and 78), 60 min (Plates 74, 75, 80 and 81), and 120 min (Plates 73 and 82 - 85).

Fig. 16 shows histograms of the lengths of silver grain tracks measured in DNA fibre autoradiographs from unsynchronized, non-FUdR-treated chick somatic cells labelled for 15, 30, 60 and 120 min. These results can be compared with the histograms from FUdR-treated chick somatic cells in Fig. 15, Chapter 3. 125 length measurements were made to compile each histogram in both Figures. The range of lengths in each histogram in Fig. 16 is: 3 - 47 μm (15 min), 4 - 123 μm (30 min), 4 - 114 μm (60 min) and 7 - 370 μm (120 min) and the mean labelled lengths are 10, 22, 29 and 47 μm, respectively. The range of lengths recorded in the histograms in Fig. 15, from cells pre-treated with FUdR, are 7 - 90 μm (15 min), 9 - 81 μm (30 min), 10 - 250 μm (60 min) and 10 - 350 μm (120 min) and the mean length in each histogram is 18, 23 51 and 104 μm respectively.

A comparison of the labelled lengths from somatic cells treated and untreated with FUdR leads to the following conclusions:

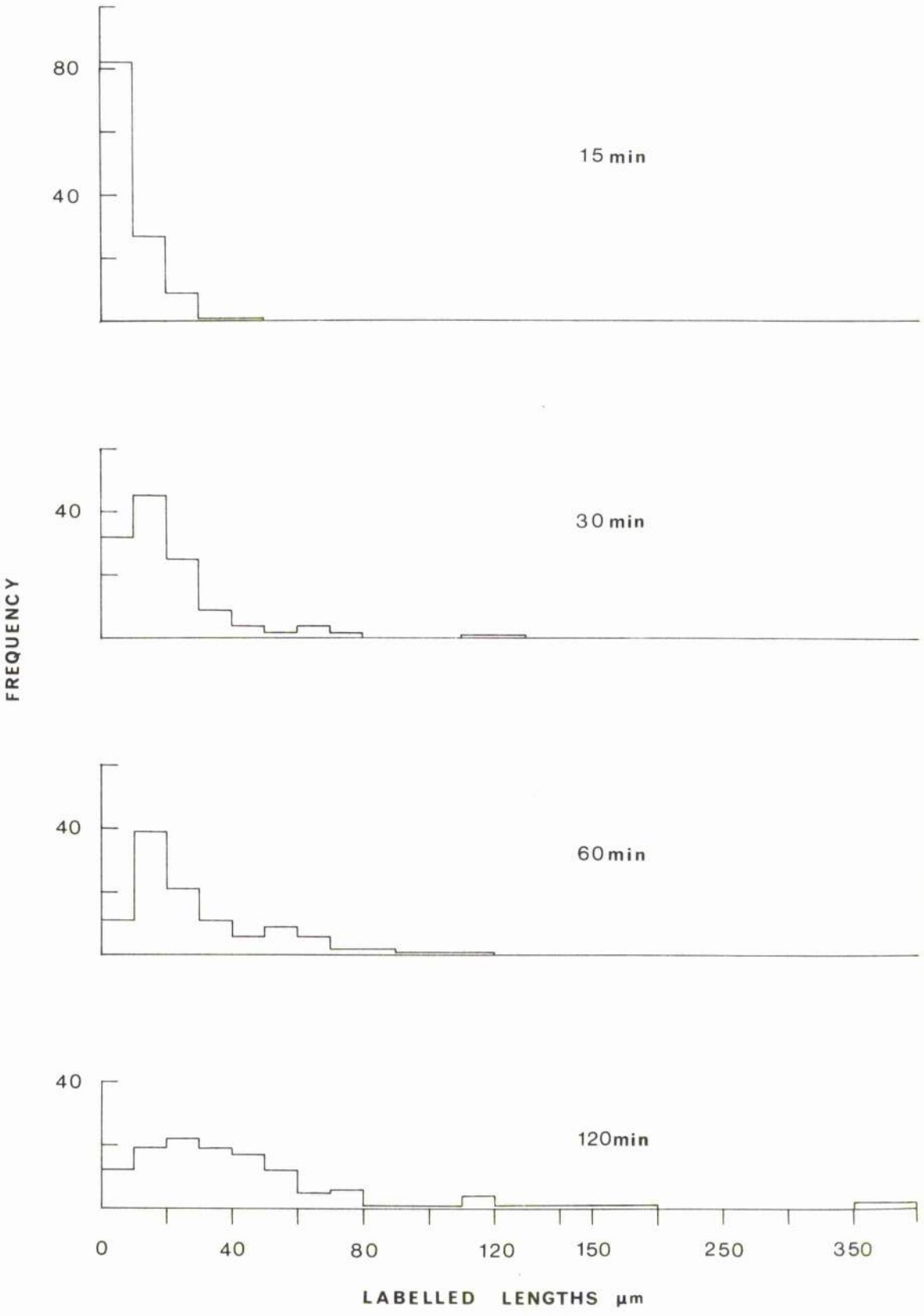
(1) In all cases, the mean labelled length recorded after the same labelling period is smaller in preparations from cells untreated with FUdR than in preparations from FUdR-treated cells.

(2) The range of lengtah recorded from cells untreated with FUdR labelled for 15 and 30 min are smaller than the comparable range of lengths recorded from FUdR-treated cells. Many more short labelled lengths are observed in preparations from untreated cells. The range of lengths is, however, similar in both types of cells after 120 min labelling.

(3) No right hand shoulder, RHS (p. 83, Chapter 3), representing those replication units that had begun replication when ^{3}H-TdR was added and had continued replication without fusion, is apparent in the histograms

Fig. 16. Frequency histograms of labelled track lengths from DNA fibre autoradiographs derived from unsynchronized chick somatic cells not pre-treated with FUdR and labelled with ^{3}H-TdR for 15, 30, 60 and 120 min.

Fig 16


80
40
15 min
40
30 min
40
60 min
40
120 min
FREQUENCY
0
40
80
120
150
250
350
LABELLED LENGTHS µm

compiled from untreated cells whereas these are clear, at least in the 15 and 30 min labelling experiments, in histograms of labelled lengths from FUdR-treated cells.

These differences may be explained by the fact that unsynchronised cells will be at various stages in the cell cycle, and therefore more replication units will have initiated before ^{3}H-TdR is added to the cells, than in a population of cells in which a third are synchronized at the beginning of S by FUdR. The labelled lengths in Fig. 16 will be not only a mixed population representing DNA replication at various times throughout S, but also a greater proportion of these lengths will represent the replication of units other than unfused two-way units than is the case in Fig. 15. Two differences are noted when Fig. 15 is compared with Fig. 16. More short labelled lengths, less than 12.5 µm after 15 min labelling, less than 25 µm after 30 min labelling, less than 50 µm after 60 min labelling and less than 100 µm after 120 min labelling are apparent in the histograms of Fig. 16. Further, fewer long labelled lengths are present in the histograms of Fig. 16. Just such differences are to be expected as a consequence of labelling unsynchronised cells.

The DNA replication rate is assessed not from the mean value nor the RHS from the 15 and 30 min histograms in Fig. 16 but from the lengths which occur with the highest frequency. This is because no distinct RHS is present in these histograms, and a mean value would represent a spread of one-way and two-way units and fusions. On the other hand, those lengths which occur with the greatest frequency should represent for the most part unfused, one-way sections. If this 'majority' value is recognised in the 15 and 30 min histograms of Fig. 16 the replication rate is evidently some 25 - 30 µm/hr one-way. This rate is the same as that

calculated for FUdR-treated chick somatic cells. If the mean initiation interval is 54 μm and the replication rate is as above, after 60 min labelling very few neighbouring one-way sections from replication units that initiated prior to labelling will have remained unfused. Hence there are few lengths of 25 to 30 μm after 60 min labelling and few lengths of 50 - 60 μm after 120 min labelling.

(v) Observation of 'sprays' of DNA

A noticeable feature of DNA fibre autoradiographs from cells untreated with FUdR is that tandem sequences of tracks sometimes occur bunched together to form 'sprays' of up to about fifty strands; within a single spray all the labelled sections are of the same or similar length. All sprays do not, show the same lengths of labelled tracks, however. Plates 86 - 90 are photographs of sprays in DNA fibre autoradiographs from cells not treated with FUdR all labelled for 60 min. Plate 86 shows a rather tangled spray of about 8 or more tandem tracks the labelled sections of which range between 28 and 100 μm, the mean being 50 μm. A tandem series of medium-short tracks is also present to the right of the photograph. Plate 87 shows a spray of about 15 or more tandem tracks the labelled sections of which range between 8 and 34 μm, the mean being 14 μm. The spray in Plate 88 is also made up of about 15 or more tandem tracks whose labelled sections range between 8 and 20 μm, the mean length being 9 μm. Two sprays of tandem tracks are shown in Plate 89, lying side by side in the same preparation. The spray at the top of the photograph is made up of about 20 or more tandem tracks, each labelled section having a mean length of 40 μm and a range of 12 to 80 μm. The bottom spray also contains 20 or more tandem tracks, each labelled

section having a mean length of 10 μm with a range of 3 to 28 μm. Plate 90 also shows two adjacently situated sprays. The top spray consists of about 10 or more tandem tracks, the labelled sections of which range between 20 and 82 μm in length with a mean being 42 μm. The bottom spray is made up of 50 or more tandem tracks with labelled sections between 4 and 24 μm in length with a mean of 10 μm. The number of strands within each spray is calculated very approximately from the number of apparently independent units or tandem series of units in parallel.

DISCUSSION

(i) Direction of replication

Various suggestions as to how FUdR might be responsible for bidirectional replication have been put forward by previous authors. Amaldi *et al.* (1972) claim that FUdR does not block DNA synthesis entirely and that this incomplete block causes an increase in the number of concurrently active initiation sites, i.e. an increase over the number that would normally be active at any one time during S in non-FUdR treated cells. This work is discussed more fully later. Amaldi *et al.* suggest that this increase may give rise to the appearance of bidirectional replication units as opposed to unidirectional ones. On the other hand, Lark et al. (1971) imply that bidirectional replication might be an artifact due to repair synthesis of breaks in the DNA caused by FUdR. Lastly, Weintraub (1972a) suggests that bidirectional replication might be caused by a build-up of initiator proteins in the cell during FUdR treatment. He states that "if initiation is regarded as a statistical event (for example, ten initiator molecules per cell lead to initiation in one direction, while twenty lead to initiations in two directions),

then bidirectional replication becomes more a reflection of the physiology of the cell than of its polymerization mechanisms".

Though only few distinct grain density shifts were observed in DNA fibre autoradiographs from non-FUdR treated cells, the fact that all such patterns were compatible with bidirectional replication but not with unidirectional replication, indicates that FUdR pre-treatment of the cells is not the cause of bidirectional replication. Huberman & Tsai (1973) arrived at a similar conclusion when they compared the replication pattern in FUdR-treated and non-FUdR treated Chinese hamster cells in culture.

(ii) Labelled lengths and replication rate

The difference between the comparable histograms in Figs 15 and 16 have been explained on the basis of semi-synchronized versus unsynchronized cells. Although this explanation is acceptable, another interpretation is possible, namely, that the increased frequency of short labelled lengths in each histogram in Fig. 16 is a result of the replication rate slowing down as S.proceeds. An argument against this proposal has already been given on p. 86, Chapter 3.

If the replication rate is 25 to 30 μm/hr one-way then lengths less than about 12.5 μm in the 30 min histogram, for example, are too short to be one-way labelled sections. Two explanations are possible, first that they represent early fusions of neighbouring one-way sections during the labelling period or secondly, many replication units that initiated late in the labelling period. This is discussed further in relation to the finding of such short units within 'sprays' of DNA.

(iii) Repair synthesis

It may also be contended that the use of an FUdR block invalidates certain conclusions about the distances between initiation points. Taylor et al. (1962) have shown that FUdR at concentrations ranging from 10^{-4} to 10^{-7} M induces breaks in Vicia faba chromosomes which are visible at mitosis but that thymidine supplied up to one hour before anaphase can prevent such breaks from appearing. This suggests that thymidine is involved in repair synthesis under these experimental conditions, and raises two questions: first, whether some of the tracks measured from cells labelled with ^{3}H-TdR after FUdR treatment (Chapter 3) represent repair synthesis, and secondly, whether some of the initiation points and therefore initiation intervals scored in DNA from ^{3}H-TdR labelled cells following the same treatment (Chapter 2) are FUdR-induced.

Until relatively recently it was believed that DNA synthesis observed in vitro corresponded to that which always occurred in normal DNA replication in vivo. It is now known that DNA synthesis may occur in vivo and in vitro not by normal replication but also as a result of repair or recombination of DNA. Repair and recombination DNA synthesis differ in their requirements and their characteristics from normal DNA replication.

If FUdR is inducing unnatural origins for replication, as a result of repair synthesis, this should be reflected in different frequency distributions of initiation intervals collected from experiments where an FUdR block was, or was not, applied, and consequential differences (because of unnaturally early fusions) in the corresponding histograms of labelled lengths. However, the 100 initiation intervals collected from non-FUdR-treated cells fall within the same range as those collected from

FUdR-treated cells and in both cases most initiation intervals lie within the range of about 35 and 75 μm, (Table 6). The difference between the histograms of labelled lengths in Figs 15 and 16 have already been explained.

(iv) Does FUdR block DNA synthesis?

Amaldi et al. (1972) have commented from an altogether different standpoint on conclusions based on experiments where FUdR has been used. Removing Chinese hamster cells from a culture by the metaphase selection procedure and immediately transferring these cells to medium containing FUdR at 4×10^{-9} M for a period longer than G_1, they found evidence for continuing though abnormally slow DNA synthesis. This was apparent in DNA fibre autoradiographs following ^{3}H-TdR labelling as frequent gaps at the mid-points of silver grain tracks. After assuming that the temporal succession of initiations is undisturbed by the FUdR treatment, Amaldi et al. claim that when the imperfect block is relieved by ^{3}H-TdR, neighbouring units are seen to be replicating coincidentally which, because of temporal staggering under ordinary conditions, would not normally be observed. This is as much as to say that 'apparent' initiation intervals collected from non-FUdR-treated cells would tend to be longer (because they would include invisible late starters) than the intervals collected from FUdR-treated cells, i.e. that FUdR-treated cells give a more accurate idea of initiation intervals! The only rejoinder I can make to this assertion is that, as already mentioned, the measurements of initiation intervals from FUdR-treated and untreated cells do not differ appreciably.

Furthermore, if DNA synthesis occurs in the presence of FUdR, although at a rate 100 times slower than normal (Amaldi et al.) and the

TABLE 7. Frequency distribution of estimated replicated lengths from FUdR-treated somatic cells labelled for 30 min and chased for 30 min expressed also as the time over which DNA synthesis has occurred. Replicated lengths were measured excluding tail regions.

Estimated replicated length (μm)	Frequency	DNA synthesis time (min)
0 - 5	0	0
6 - 10	1	0
11 - 15	0	0
16 - 20	3	16 - 24
21 - 25	10	21 - 30
26 - 30	2	26 - 36
31 - 35	3	31 - 42
36 - 40	4	36 - 48
41 - 45	4	41 - 54
46 - 50	2	46 - 60
51 - 55	3	51 - 66
56 - 60	2	56 - 72
61 - 65	3	61 - 78
66 - 70	2	66 - 84
71 - 75	1	71 - 90
76 - 80	1	76 - 96
81 - 85	1	81 - 102
86 - 90	0	0
91 - 95	0	0
96 - 100	0	0
101 - 150	3	101 - 180
151 - 200	1	151 - 240

temporal order of initiations remains unchanged, then in the present study, cells labelled after FUdR pre-treatment for 16 hr (Chapters 2 and 3) should show the following characteristics. (i) All replication units from cells in S, or from cells in G_1, M or late G_2 which enter S during the time the cells are in FUdR will have been activated. This is estimated because S lasts 7.5 hr, G_1 about 8.75 hr, G_2 about 4 hr (Figs 1 and 2, Chapter 1) and M probably about 1 hr (Cleaver, 1967). Only those cells in early G_2, about 20% of the cell population, will not have activated the total number of their replication units. (ii) Many cells thought to be blocked at the G_1/S interphase by FUdR i.e. 33% (see p. 37 and p. 77) will be at some later stage in S and therefore will also have activated many of their replication units prior to labelling. This means that few labelled sections representing two-way units which initiated immediately ^{3}H-TdR was applied should be evident in DNA fibre autoradiographs from chick somatic cells, pre-treated with FUdR for 16 hr.

The frequency with which such uninterrupted, two-way labelled sections that initiated immediately ^{3}H-TdR was available occur in DNA fibre autoradiographs from FUdR-treated cells labelled for 30 min and chased for 30 is estimated in Table 7. It is assumed that termini do not exist (p. 73, Chapter 2) and that the replication rate is 25 - 30 µm/hr one-way. The measurements in Table 7 are derived from those previously used in Fig. 10, Chapter 2. In this analysis the replicated length of 46 replication units was assessed by measuring the length of 46 heavily labelled sections (either uninterrupted or showing a mid-gap) flanked at both sides by tails (See p. 51, Chapter 2) in DNA fibre autoradiographs from FUdR-treated cells labelled for 30 min and chased for 30 min. These lengths are each converted in Table 7 to measurements of their total time spent in replication from initiation to harvesting of

the cells, from the known value of DNA replication rate. The results demonstrate that 33% (15 examples in 46) correspond to the expected two-way replicated length if initiation had occurred immediately ^{3}H-TdR was applied. 6% of the replication units must have initiated some time after ^{3}H-TdR was applied and 61% of the replication units had mid-gaps and therefore initiated before ^{3}H-TdR was applied to the cells.

This analysis shows that a significant proportion of the observed labelled lengths, 33% in this case, initiate immediately the FUdR block is released by the adding of ^{3}H-TdR, but not earlier, when the cells are in the presence of FUdR.

(v) Sprays of DNA

The sprays of DNA mentioned on p. 99 have not been observed in DNA fibre autoradiographs from FUdR-treated chick somatic cells.

Before consideration of these sprays it must be remembered that labelled sections in tandem often vary considerably in length in preparations from both cells treated and untreated with FUdR (described in Chapters 2 and 3, and Plates 65 to 85 this Chapter). Sprays of tandemly arranged labelled sections of similar length are therefore the exception, not the rule. In fact they have only been detected with any frequency in preparations from cells untreated with FUdR and labelled for 60 min. The following interpretations are for this reason only true of a small proportion of the DNA observed in the DNA fibre autoradiographs from chick somatic cells untreated with FUdR.

These sprays may be interpreted in either of two ways. First, each spray might represent the DNA replicated at any one time by a single

cell, and this DNA is not dispersed over the millipore filter but remains bunched together spraying out from its lysed nucleus. This should occur in preparations from both FUdR-treated and non-FUdR-treated cells because the later experimental procedure is identical in both cases. If, however, sprays of differing units length represent DNA from cells at different stages in S, this would explain why such sprays are only readily apparent in preparations from unsynchronized cells. Secondly, each spray may represent the replication of DNA from different cells at different stages in S, but where each spray represents the replication of only a small percentage of the genome within each cell. All sprays may then be the same 'type' of DNA at various stages in its replication. Thirdly, two or more adjacently situated sprays that show different labelled lengths may represent DNA that is from a single cell but is spatially separate. The different labelled lengths might then reflect the time during S at which the replication units in each spray initiated. The labelled lengths observed after labelling a cell synchronized at the beginning of S, for example, for 60 min, will necessarily **all** represent fused or unfused two-way units. The labelled lengths from a cell labelled in mid or late S, however, will represent a mixed population of one and two-way sections and various types of fusions. Clearly, such sprays would therefore be more obvious in DNA fibre autoradiographs from unsynchronized cells than from semi-synchronized cells.

The second or third interpretations seem most likely because the amount of DNA within each spray, (in the region of 1 cm, on average) is far less than the amount of DNA within each diploid cell (about 1 M) even allowing for the fact that only one-seventh of the DNA within a cell might be replicated at any one time (See p. 87, Chapter 3).

The question of why all the labelled sections within a single spray should be of similar length may now be discussed.

(1) One explanation might be that such sections represent unfused, one-way units from replication units that had initiated before ^{3}H-TdR was supplied to the cells. This interpretation seems likely as many one-way labelled sections are expected in preparations from unsynchronized cells. Labelled lengths formed by two-way replication may be absent because many sprays represent DNA replication in late S i.e. all replication units might have initiated before ^{3}H-TdR was available. Intermediate labelled lengths may be absent if fusions of neighbouring replication units only occurred before or after ^{3}H-TdR was applied or if any fused labelled lengths were coincidentally similar in length to the one-way labelled sections characteristic for that spray.

A discrepancy remains. If short labelled lengths, for example, those in Plate 88 and the lower sprays in Plates 89 and 90 represent unfused one-way labelled lengths (plus fused lengths coincidentally the same length) then the distance between initiation points must, in nearly all cases, be greater than 60 µm. This follows if the replication rate is 25 - 30 µm/hr one-way and the cells were labelled for 60 min. Treating the labelled tracks as one-way units, initiation intervals may be measured approximately from the distance from the mid-gap separating one pair of labelled sections, both of equal length, to the mid-gap separating a neighbouring pair of labelled sections again of equal length. In Plate 88, for example, most initiation intervals measured in this way are not greater than 60 µm but about 50 µm or less, a slightly lower estimate than the mean initiation interval shown in Fig. 16, 54 µm. This means that the replication rate of the DNA within such a spray must

be far less than 25 to 30 μm/hr one-way if the labelled sections represent unfused one-way units. The mean labelled length in Plate 88 is 10 μm which would suggest a replication rate of only 10 μm/hr one-way. Furthermore, if sprays with longer mean labelled lengths as in Plate 86 also represent unfused one-way sections, the replication rate of the DNA must vary between individual cells. This, in my opinion, is unlikely (See also p. 86, Chapter 3).

(2) An alternative explanation might be that such sections represent two-way replication units that initiated more or less synchronously during the period label was available. This is supported by the evidence in Fig. 10, Chapter 2, a correlation showing a tendency for neighbouring initiation sites to initiate near synchronously in FUdR-treated somatic cells. It has been noted in preceding chapters that activation of replication units must be staggered throughout S. Given that these labelled lengths do represent two-way replication units, that the cells are unsynchronized, and that replication units within a spray initiate synchronously, then activation of initiation sites would seem to occur in bursts throughout S.

(3) Another explanation might be that the labelled sections within a spray are the same length because they represent fused, neighbouring one-way sections, if all such fusions had occurred at the same time in S in synchrony sometime during the labelling period all such replication units having initiated prior to labelling though not necessarily in synchrony. This possibility would seem, however, to be remote because of its complexity.

(4) Finally, labelled sections within a spray might be similar in length if the labelled sections represented two-way units where replication was terminated about an origin at defined termini, all concurrently active units within each spray being uniform in length. Sprays of short labelled lengths may then represent units that initiated some time during the labelling period, all reaching their termini during the period of labelling, but not necessarily all initiating in synchrony. Sprays of long labelled sections must, however, represent replication units that initiated at or near synchronously during the labelling period. The argument against this proposal is that the presence of defined termini has already been challenged in Chapter 2.

The most likely explanation would therefore seem to be that such tracks represent two-way units which initiate in synchrony within each spray. Sprays of very short labelled sections, as in Plate 88 and the bottom sprays in Plates 89 and 90, might represent replication units that initiated in synchrony late in the labelling period. Sprays of relatively short lengths, as in Plate 87, would then represent replication units that initiated in synchrony slightly earlier in the labelling period. If the labelled sections within such sprays represent two-way units then the distances between initiation points may be measured from the mid-point of a labelled section to the mid-point of a neighbouring labelled section in tandem. Intervals measured in this way in Plate 88, for example, are between 20 and 40 μm, the mean being some 33 μm, a great deal shorter than the mean established from Fig. 16. That the distances between initiation sites are small within sprays agrees with the observation that labelled sections within sprays are often in clusters, implying closely spaced initiation sites. If the replication rate is some 25 to 30 μm/hr one-way, neighbouring replication units that had initiated

in synchrony near the beginning of the labelling period would be expected to give rise to long labelled lengths, representing fusions, after 60 min of labelling. Sprays of long labelled lengths in Plate 86 and the upper half of Plate 89 and 90 may be the result of such clusters of closely spaced replication units initating and fusing during the labelling period. The presence of unlabelled gaps between long labelled sections implies that within a spray not all the DNA is replicated together.

The question that now remains is what such sprays might reflect. First, the fact that they are only obvious in DNA fibre autoradiographs from unsynchronized chick somatic cells suggests that they are characteristic of the DNA which is synthesized later than during the first two hours of S. Sprays of short labelled lengths would only be noticeable when the majority of labelled sections in an autoradiographic preparation were long. Sprays of long labelled lengths would only be evident after relatively long periods of labelling when clusters of neighbouring two-way sections could have initiated and fused in the presence of label. This would explain why such sprays are not evident in cells untreated with FUdR labelled for 15 and 30 min. Secondly, the replication units within a spray have closely spaced initiation sites which are activated synchronously or nearly so. This implies that the DNA being replicated within such sprays must be fast-replicating.

It has already been suggested in Chapter 2 that clusters of tandem replication units having similar initiation intervals and similar initiation times may represent the replication of a 'type' of DNA. Clusters with different characteristics would then represent the replication of different DNA types.

Two sprays which have labelled sections of different length may represent the replication of two types of DNA. Alternatively, all sprays may represent the replication of one and the same type of DNA, the varying lengths between sprays indicating that the DNA within each spray was from different cells and at a different stage in its replication.

In whole cell autoradiographs satellite DNA has been found to be late-replicating in almost all species studied (Bostock & Prescott, 1971; Flamm *et al.*, 1971). 5% of the DNA in the chicken genome is satellite DNA (Comings & Mattoccia, 1972a). It is not known whether this DNA is late-replicating in the chicken, but this seems likely.

Lima-de-Faria & Jaworska (1968) have presented a summary of the time of replication of euchromatin and 'heterochromatic' regions of chromosomes as found in a number of different plant and animal systems. With few exceptions it was noted that heterochromatic regions replicate their DNA late in S. Comings & Mattoccia (1972a) found that there was an increase in the amount of repetitious satellite DNA in heterochromatic fractions in the chicken. This supports the suggestion that satellite DNA is late-replicating in the chicken.

Priest (1968) has shown that the heterochromatic X chromosome of a human cell replicates faster than human euchromatic chromosomes. This may indicate that heterochromatic regions of chromosomes in other animals are also characteristically fast-replicating. That late-replicating regions in human chromosomes show higher rates of synthesis has also been shown by Gilbert *et al.* (1965) and Gavosto *et al.* (1968).

The above information suggests that the sprays of DNA observed in these autoradiographs which are characteristically both late and fast replicating may well come from heterochromatic chromosomes or regions of chromosomes, the DNA of which is in the form of satellite sequences.

This means that satellite DNA would be replicated here by units with relatively uniform characteristics, different from the units involved in the remainder of the genome.

Birds possess unusually small chromosomes called microchromosomes (Plate 3). Arrighi & Stefos (1971) have used a denaturation/renaturation staining method to detect heterochromatin and have found that microchromosomes of a number of birds including the chicken contain heterochromatin whilst macrochromosomes contain very little. In situ hybridization studies by Brown & Jones (1972) have shown that the satellite sequences are located predominantly in the microchromosomes in the Japanese quail and it is likely that this holds for other birds, including the chicken. The DNA within the microchromosomes represents only a very small percentage of the total genome, at a rough estimate from inspection of metaphase plates some 15%. From this it follows that satellite sequences would be expected to be localized in long stretches, not interspersed in short sections between long stretches of non-repetitious DNA. If the sprays represent satellite DNA then it follows that within each spray the replication units would be remarkably uniform. The localization of satellite DNA in the microchromosomes of the chicken may be questioned, however, because these chromosomes have not been found to be late replicating by Schmid (1962) and Bianchi & Molina (1967).

That the sprays do represent the replication of satellite DNA is supported, however, by other workers. Hori & Lark (unpublished) have recently found evidence that the satellite DNA in the kangaroo rat, Dipodomys ordii is replicated by much shorter replication units than is the rest of the DNA of this species. The satellite DNA, as in the 'sprays' observed in this study, may be recognized in DNA fibre

autoradiographs from whole cells because it is clumped together and made up of particularly short labelled sections.

Possibly there are other regions of fast-replicating DNA which also consist of repetitious sequences but interspersed between lengths of DNA that is neither highly repetitious nor heterochromatic. Where clusters of closely spaced neighbouring units are seen to be replicating concurrently these may indicate the position of intercalated fast-replicating DNA. Such clusters are shown in the lower half of Plate 56, from somatic cells treated with FUdR, in the lower halves of both Plates 73 and 69 and in the centre of Plate 70, from cells untreated with FUdR and to the left of the sequence in Plate 101, from cells isolated from blastoderm tissue.

CHAPTER 5.

DNA REPLICATION IN THE BLASTODERM

INTRODUCTION

The term blastoderm is applied to the chick embryo from the first cleavage stage to the first formation of somites, after about 23 to 26 hours of incubation at 38°C (Hamburger & Hamilton, 1951). The 18 hr-embryo (37°C) was chosen for study for the following reasons. First, the embryo before this time is extremely fragile and disintegrates on the slightest handling. Secondly, the embryo "proper" begins to form with the rising of the head fold and the formation of the first somite after some 25 hours of incubation at 37°C. An 18 hr-embryo at 37°C represents a Stage 3/4 embryo as described by Hamburger & Hamilton; the primitive streak is usually quite long and the area pellicida is not yet pear-shaped. This means that at stage 3/4 the germ layers are still forming, i.e. the hypoblast and epiblast are separating and the primitive streak is being generated by the invagination of cells from the hypoblast to form the mesoderm layer and from the rate of increase in size of the blastoderm the cells would appear to be proliferating rapidly. It was supposed that as these cells have short generation times, their S-phases would probably be shorter than those of somatic cells, and that this would be reflected in DNA fibre autoradiographs. Plate 2 shows a chick embryo isolated after 18 hours incubation at 37°C.

Most embryonic tissues have shorter S-phases than their adult tissues (see work cited by Mitchison, 1971), and it was assumed on beginning this study that the chick blastoderm would not be an exception. From work carried out by Callan (1972, 1973) the S-phase in *Triturus*

blastulae was found to be about 1 hr at 18°C, i.e. one fiftieth the duration of S in somatic cells. Callan discovered that this quantitative increase in DNA replication rate was accommodated by an increase in the number of concurrently active initiation sites, and a reduction in initiation interval distance. DNA fibre autoradiographs were prepared from chick blastoderms to see whether the same compensatory features might occur in this organism. It was found, however, that the replication patterns in blastoderm and somatic cells is similar. A determination of S-phase duration in the 11½ to 27 hr old blastoderm thereafter demonstrated that the S-phase is about 5 hr (Fig. 3). The S-phase duration in chick somatic cells in vitro is 7.5 hr (Fig. 1). A similar replication pattern for both cell types is therefore to be expected.

METHOD

18 hr-old blastoderms were dissected from eggs according to the method outlined in the General Account of Materials and Methods. In some experiments embryos were isolated into growth medium containing FUdR and uridine. These embryos were incubated in this medium for 2 hr and then transferred into growth medium containing ^{3}H-TdR. Other embryos were isolated directly into labelled medium without FUdR pre-treatment. 5 embryos, about 5 x 10^{6} cells, were labelled in each experiment. The blastoderms were labelled according to three schedules: (i) 30 min in high specific activity ^{3}H-TdR; (ii) 60 min in high specific activity ^{3}H-TdR; (iii) 30 min in high specific activity ^{3}H-TdR followed by a 30 min chase in the same medium supplemented with non-radioactive thymidine at three times the concentration of the high specific activity ^{3}H-TdR (29 Ci/mMol, 50 µCi/ml) previously added. Labelling was terminated in each experiment by bringing the cells to 0°C. DNA fibre autoradiographs

were prepared and left to expose for 7 months. The full age of 18 hr-incubated blastoderms treated with FUdR is 20 hr. The blastoderms are referred to, however, by their age at isolation, i.e. 18 hr-old.

RESULTS

(i) Thymidylate pool

DNA fibre autoradiographs prepared from blastoderms isolated *in vitro* after 18 hr incubation and labelled directly without FUdR pre-treatment were unsuccessful because few labelled molecules were incorporated into the nascent DNA. After 7 months exposure the grain density along tracks which appeared to represent strands of DNA was extremely low. It is therefore presumable that the pool of thymidylate molecules is very high in these early embryonic cells, higher than the pool in somatic cells. Pre-treatment of blastoderms with FUdR for 2 hr greatly increases the grain density in DNA fibre autoradiographs. Plates 91 to 107 are photographs of tandemly arranged labelled sections in DNA fibre autoradiographs prepared from blastoderm cells pre-treated with FUdR for 2 hr. Plates 91 - 92 and 101 - 103 are from cells labelled for 30 min, Plates 93 - 95, 99 - 100 and 104 are from cells labelled for 60 min and Plates 96 - 98 and 105 - 107 are from cells labelled for 30 min and chased for 30 min. As in somatic cells treated with FUdR, DNA is replicated at tandemly arranged initiation sites and the labelled sections increase in length with increased duration of labelling.

Tails are not very obvious in the labelled sections from chased preparations. This is presumably because the cells own thymidylate pool swamps the number of incoming ^{3}H-TdR molecules during the first 30 min pulse to such an extent that there is no distinct demarcation between

heavily labelled DNA synthesised during the initial high specific activity pulse and lightly labelled DNA synthesized during the subsequent chase period.

Where tails can be recognised the pattern is in accordance with bidirectional replication (See Fig. 4, Chapter 2).

(ii) Labelled lengths and replication rate

The labelled sections from DNA fibre autoradiographs from chick blastoderms labelled for 30 and 60 min are similar to the lengths of labelled sections in DNA fibre autoradiographs from chick somatic cells labelled for 30 and 60 min, the similarity being especially true of blastoderms when compared with somatic cells not treated with FUdR.

Lengths of labelled sections from chick blastoderms pre-treated with FUdR and labelled for 30 and 60 min are shown as histograms in Fig. 17. After 30 min labelling the mean labelled length is 16 μm and the majority of lengths are 5 - 30 μm. The majority of labelled lengths from somatic cells pre-treated with FUdR and labelled for 30 min are 10 - 40 μm (See Fig. 15, Chapter 3). If the somatic cells are unsynchronized, however, the majority of labelled sections are between 5 and 30 μm, after 30 min labelling (Fig. 16, Chapter 4). After 60 min labelling, the mean labelled length in blastoderm preparations is 34 μm and most lengths are 5 - 50 μm. The majority of labelled lengths from somatic cells pre-treated with FUdR and labelled for 60 min are 10 - 70 μm, and from somatic cells untreated with FUdR and labelled for 60 min, 5 - 40 μm.

The S-phase in the 11½ - 27 hr blastoderm is about 5 hr and $G_1 + G_2 + M$ is some 4 hr (Chapter 1 and Fig. 3). Blastoderm cells were

heavily labelled DNA synthesized during the initial high specific activity pulse and lightly labelled DNA synthesized during the subsequent chase period.

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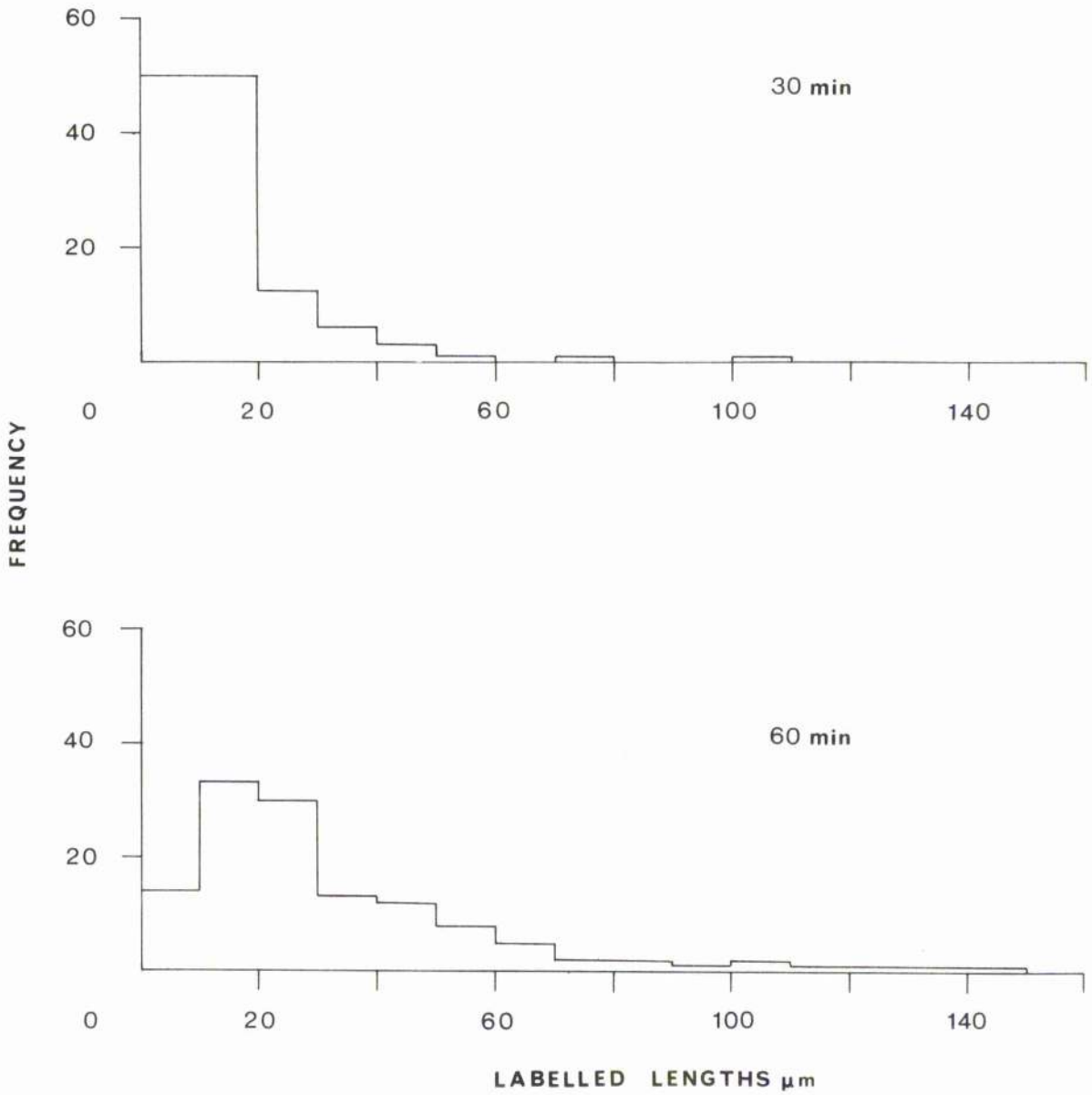
The S-phase in the $11\frac{1}{2}$ - 27 hr blastoderm is about 5 hr and $G_1 + G_2 + M$ is some 4 hr (Chapter 1 and Fig. 3). Blastoderm cells were

Fig. 17. Frequency histogram of labelled track lengths from DNA fibre autoradiographs derived from chick blastoderms incubated for 18 hr, isolated *in vitro*, treated with FUdR and labelled with ^{3}H-TdR for 30 and 60 min.

Fig 17


60
40
20
0
20
60
100
140
30 min
60
40
20
0
20
60
100
140
60 min
FREQUENCY
LABELLED LENGTHS µm

pre-treated with FUdR for 2 hr to reduce their thymidylate pool, not to synchronise a large percentage of the cells. Clearly, pre-treatment of these cells with FUdR for only 2 hr would synchronise less than 20% of the cells at the beginning of S. For this reason many unfused one-way sections from bidirectional replication units that initiated before ^{3}H-TdR was supplied will be labelled, especially after short periods of labelling. It is not surprising, therefore, that the histograms in Fig. 17 resemble the histogram of labelled lengths from somatic cells not treated with FUdR rather than the histograms of labelled lengths from FUdR-treated somatic cells.

A right hand shoulder is not very obvious in the histograms in Fig. 17 as also in the histograms of labelled lengths from cells untreated with FUdR. A value for the replication rate of DNA in the blastoderm can be found as in the latter, from the lengths of the "majority" of labelled sections in the 15 and 30 min histograms. The majority rather than the mean length is taken because it is anticipated that the majority represents mainly unfused one-way sections, as is likely after short periods of labelling. The majority of labelled lengths after 30 min labelling are between 5 and 10 µm, if such lengths are mainly one-way unfused sections, the DNA replication rate must be some 10 to 20 µm/hr one-way. Similarly, the majority of labelled lengths after 60 min labelling are between 15 and 25 µm which suggests a replication rate of some 15 to 25 µm/hr one-way. The difference in length between the majority of lengths in the two histograms also gives an estimate for the rate of replication over 30 min, some 10 - 30 µm/hr one-way. The overall value for the replication rate is therefore about 10 - 30 µm/hr one-way.

TABLE 8. Frequency distribution of intervals between initiation points for DNA replication derived from 18 hr-incubated chick blastoderms, pre-treated with FUdR for 2 hr, labelled with ^{3}H-TdR for 30 min or labelled with ^{3}H-TdR for 30 min and "chased" for 30 min.

"Chased"		"Unchased"	
initiation interval/μm	frequency	initiation interval/μm	frequency
0 - 10	0	0 - 10	0
11 - 20	0	11 - 20	0
21 - 30	0	21 - 30	2
31 - 40	2	31 - 40	6
41 - 50	5	41 - 50	5
51 - 60	6	51 - 60	2
61 - 70	7	61 - 70	4
71 - 80	4	71 - 80	3
81 - 90	2	81 - 90	2
91 - 100	1	91 - 100	1
101 - 110	1	101 - 110	1
111 - 120	0	111 - 120	2
121 - 130	0	121 - 130	1
131 - 140	1	131 - 140	1
141 - 150	0	141 - 150	0
151 - 160	0	151 - 160	0
161 - 170	1	161 - 170	0
TOTAL	30		30
MEAN	69		66

The DNA replication rate in somatic cells has been determined as 25 to 30 μm/hr one-way. The range is greater in the above determination for blastoderms. This disparity makes it impossible to say emphatically that replication rate is the same in both cell types rather than that it is somewhat slower in the blastoderm, as is the situation in Triturus embryos. Overall similarity is the general conclusion to be drawn.

(iii) Initiation intervals

Intervals between initiation points were measured from 30 min labelled/30 min chased material as in Chapter 2. As previously mentioned tails were often difficult to recognise in these DNA fibre autoradiographs from pulse-chased cells. Initiation intervals were, therefore, also measured from unchased, 30 min labelled preparations. Where neighbouring labelled sections were of equal length these were taken to be the two one-way units of a replication unit that had initiated prior to labelling at the mid-gap between such units. Initiation intervals were measured as the distance from mid-gap to mid-gap between two such neighbouring pairs of labelled sections arranged in tandem. The results from both series of measurements are shown separately as frequency distributions in Table 8. It was only possible to measure 30 intervals accurately from both sets of preparations. The range of initiation intervals measured from 30 min-labelled/30 min chased preparations is 30 to 170 μm, the mean equals 69 μm, and most intervals are between 45 and 70 μm. The range of intervals from 30 min-labelled preparations is 25 to 140 μm, the mean equals 66 μm and the majority of intervals are between 35 and 70 μm. Neither range nor mean are strikingly different using either procedure. It is unsatisfactory that such a small number of determinations could be made; nevertheless, the overall mean, 68 μm can be considered an

approximate value. The mean initiation interval from non-FUdR-treated somatic cells is 54 μm (Fig. 16) and the range 18 to 150 μm. The mean initiation interval from FUdR-treated somatic cells is 63 μm and the range 25 to 145 μm. Neither the range nor the mean distance between neighbouring initiation sites determined from 18 hr old blastoderms is very different from that found in somatic cells, with or without prior FUdR treatment.

DISCUSSION

Before the replication pattern in blastoderm and somatic cells is compared a question to be considered is whether the 5 hr S-phase calculated for the 11½ to 27 hr blastoderm (Chapter 1) truly represents a shorter S-phase than the 7.5 hr calculated for somatic cells. There are several arguments against this difference being significant. First, the counting of small populations of labelled cells in both techniques always means that great variations can occur between data from different experiments (See, for example, a review of S-phase determination in Cleaver, 1967). Secondly, the estimation of S-phase duration in the blastoderm may only be considered as a rough approximation because no account was taken of the fact that the blastoderm must represent an exponentially growing population of cells. Thirdly, experimental differences made it essential for the S-phases in both experiments to be determined using different techniques. This in itself may result in a variation in the data, for different levels of accuracy must occur between techniques. Fourthly, as mentioned in Chapter 1, the S-phase calculated in the present study for chick somatic cells is longer than previous estimates, (Cameron, 1964; Fujita, 1962; Basaleer, 1968; Liebecq-Hutter, 1965) where S *in vitro* and *in vivo* was found to be some 5 to 6 hr. This demonstrates that significant variations in the values for S-phase duration determined in the same tissue do occur; they may be due to inherent variations in the cells, or to variations in techniques.

When blastoderms are cultured *in vitro* do the cells retain their blastoderm characteristics, or do they take on the characteristics of fibroblasts in tissue culture? If the latter is true, this would explain the similarity in S between the two cell types. Against this argument are two considerations. (1) Blastoderms isolated *in vitro* at the time of primitive streak formation can continue developing normally at the same rate as that observed *in vivo* until about the 20 somite stage, i.e. after 50 hours incubation at 37°C (New, 1955); although the blastoderms were not always kept intact in the experimental procedure used in the present study it is probable that at least over the first few hours *in vitro* the cell cycle and DNA replication is similar to that *in vivo*. (2) According to Emanuelsson (1965) T increases rapidly in the early blastoderm *in vivo* (T = 2.1 ± 0.5 hr just prior to laying and 7.4 ± 2.0 hr at about the moment of laying). If, as is likely, S increases proportionally with T while development proceeds, then after about 18 hr incubation T *in vivo* will be somewhat greater than 7.4 ± 2 hr and an S-phase in the order of 5 hr is to be expected.

The determination of S-phase duration and the labelling of cells for DNA fibre autoradiography was carried out in blastoderms *in vitro* rather than *in vivo*, because experimentally it was much simpler and because a direct comparison could then be made with the *in vitro* analysis of S in somatic cells.

The replication rate in 18 hr-incubated blastoderms is some 10 to 30 µm/hr one-way, according to Fig. 17. Because blastoderm cells treated with FUdR for 2 hr are virtually unsynchronized, many labelled sections will represent one-way units. This 'majority' value is taken to calculate the replication rate because no RHS is present in either histogram and

and the mean value would include two-way labelled sections, fusions and late starters. The large proportion of labelled sections below about 10 μm in the 30 min histogram and below about 20 μm in the 60 min histogram suggest that the DNA replication rate in the blastoderm may be less than that calculated for chick somatic cells, 25 - 30 μm/hr one-way, if such sections represent unfused one-way units. Such lengths could, however, represent two-way replication units that initiated late in the labelling period or, as is most likely, the fusion of converging one-way sections that occurred early in the labelling period. This increase in the proportion of labelled lengths shorter than those anticipated if they represented one-way, unfused units is also seen in the histogram of labelled lengths from non-FUdR-treated somatic cells (Fig. 16). If this interpretation is correct, the lower value for the rate of DNA replication in the blastoderm (about 10 μm/hr one-way) is not a valid estimate.

The mean initiation interval in the blastoderm is 68 μm (Table 8). The mean initiation interval in somatic cells untreated with FUdR is 54 μm (Table 6, Chapter 4) and in FUdR-treated somatic cells, some 63 μm (Fig. 7, Chapter 2). Although the mean value is slightly greater in the blastoderm I suspect this difference to be a result of the paucity of examples that could be measured in the blastoderm rather than a valid increase.

If the S-phase is in fact shorter in the blastoderm and the mean initiation interval and DNA replication rates are the same in both blastoderm and somatic cells, the only way in which the DNA could be replicated within a shorter time is for the degree of staggering in the time of initiation to be less in the blastoderm. Because only slight compensation would be required, it is unlikely that it would be detected

in DNA fibre autoradiographs. In practice, however, the reverse is true, namely long gaps between tandem labelled tracks are more frequently observed in DNA fibre autoradiographs from chick blastoderms than from chick somatic cells (ref. Plates 99, 106 and 107, and compare with the photographs of DNA fibre autoradiographs described in Chapters 2, 3 and 4). Such long gaps imply a great deal of staggering in the time of initiations of neighbouring clusters of replication units.

SUGGESTIONS FOR FURTHER WORK

DNA fibre autoradiography is an elegant technique and it merits further exploitation.

(1) It would be of interest to confirm, using other species including the chicken, the work carried out by Callan (1972) on *Triturus*. He found that the sizes of the units involved in replication, and the amount of staggering in the timing of their initiations, differs in meiotic, somatic and embryonic cells in direct relationship to the gross differences in S-phase duration in these cells. Although the S-phase duration in the chick blastoderm after some 12 hours incubation has been shown to be similar to that found in somatic cells, younger embryos within the oviduct prior to laying have much shorter S-phases. Emanuelsson (1965) calculates that a 500 cell blastoderm *in vivo* has a cell generation time of 1.75 ± .25 hr. DNA fibre autoradiographs prepared from these young embryos could provide evidence as to whether or not Callan's original findings apply to other eukaryotes. DNA synthesis in mammalian embryos has not yet been investigated by this technique; there is plenty of information from somatic cells in culture with which comparisons might be drawn.

(2) Another question arising from the present study is whether the pattern of DNA replication throughout S, and therefore the sequential replication of specific parts of the genome, varies between different tissues or cell types. Changes in the replication pattern may well be related to cell differentiation.

(3) To my mind, however, the most important avenue along which this research could proceed would be an investigation of the relationship

between replication units and 'type' of DNA, for example, satellite DNA, heavy shoulder DNA or "heterochromatin", and probing deeper, the relationship between replicational and transcriptional units, such as nucleolar organizer DNA.

The answers to these questions could be pursued along several different lines, in some cases continuing the investigation in birds.

It is not known why birds, monotremes and some reptiles have small microchromosomes, about 1 μm or less (Plate 3). In the Japanese quail these microchromosomes are known to be late-replicating, heterochromatic, nucleolus-organizing and enriched in heavy shoulder and satellite DNA (Comings & Mattoccia, 1972b; Brown & Jones, 1972). DNA fibre autoradiographs of synchronized Japanese quail cells prepared from cells near the end of S should show the replication of predominantly microchromosomal DNA. It is possible that this DNA might show characteristics (such as the sprays of labelled sections of equal length as already noted in the chicken in Chapter 4) different from DNA replicated early in S. I have already begun preliminary investigations along these lines.

Chicken microchromosomes do not appear to be late replicating (Schmid, 1962; Bianchi & Molina, 1967) nor heterochromatic according to Ohno (1961) although Arrighi & Stefos (1971) contradict this finding. Like Japanese quail they are nucleolar-organizing and enriched in heavy shoulder DNA (Comings & Mattoccia, 1972b). If microchromosomes could be separated from the large chromosomes after labelling, a direct investigation of their replication could be made. In a preliminary way I have attempted to use the chromosome separation procedure outlined by Maio & Schildkraut (1967, 1969) and the method for isolating nucleoli outlined by Schildkraut & Maio (1968). This latter technique was used on the basis

that microchromosomes are nucleolus-organising and would be separated along with nucleoli.

DNA replication in chicken and Japanese quail microchromosomes could also be compared, and further attempts made to determine whether or not chicken microchromosomes are heterochromatic. To this end I have already carried out some diagnostic staining reactions. If chicken microchromosomes prove to be non-heterochromatic, as has been claimed, then differences between the replication pattern of chicken and quail microchromosomes might provide a guide as to the relationship between the 'packing' of chromatin and the pattern of DNA replication.

A more direct attack on this problem would follow if DNA could be fractionated into various components such as main band, heavy shoulder, and satellite in such a way that relatively long, unsheared lengths could be harvested. Cells could be labelled with ^{3}H-TdR according to various protocols, the DNA fractionated and then fibre autoradiographs prepared from the individual fractions. Hori & Lark (unpublished) have very recently outlined such a method whereby satellite DNA is isolated in lengths of about 100 µm from main band DNA in the kangaroo rat, Dipodomys ordii. These authors have produced DNA fibre autoradiographs of isolated satellite DNA, and have demonstrated that this DNA is characterised by very short tandemly arranged replication units.

SUMMARY

1. DNA fibre autoradiography has been used to study the replication of chromosomal DNA from chick somatic cells in tissue culture at 37°C, with or without FUdR pre-treatment, and from FUdR-treated chick blastoderm cells.

2. Replication proceeds as first described by Huberman & Riggs (1968). Tandemly arranged units replicate bidirectionally from fork-like growing points, sister strands replicating concurrently. Evidence against the existence of defined termini is presented.

3. The rate of replication in these chicken cells is some 25 to 30 µm/hr one-way. The mean interval between adjacent initiation sites for replication is similar in all three cell types but is most accurately determined from FUdR-treated chick somatic cells, being 63 µm, range 25 to 145 µm.

4. This mean value is either within the range or as much as twice as long as estimates obtained by other authors for various mammals. C-values in the chicken and mammalian species are 1.45 and about 3 pg respectively. Clearly in this comparison the mean initiation interval is not related to C-value.

5. The S-phase is estimated to be 7.5 hr in chick somatic cells and 5 hr in the blastoderm. The replication pattern is much the same in both and is therefore related to S-phase duration, not cell type. From values of replication rate, mean initiation interval and S-phase, it is clear that only about one-seventh of the DNA is replicated at any one time during S in somatic cells.

6. It has been claimed that FUdR does not completely block DNA synthesis, and that it promotes unnatural initiation points from which repair synthesis proceeds. However FUdR as used in the experiments described in this thesis has neither of these effects.

7. Although initiation intervals and the times of initiations vary, neighbouring intervals tend to be similar in length and neighbouring units tend to initiate together. Clusters of concurrently replicating, neighbouring units are bordered by long stretches of DNA which are replicated at different times.

8. Autoradiographs from unsynchronized somatic cells show "sprays" of closely arranged tandem labelled tracks, in which the track lengths are remarkably uniform. It is suggested that these sprays represent the replication of satellite DNA.

ACKNOWLEDGEMENTS

It is a great pleasure to thank those people who have contributed to the progress of this research during the past three years. In particular, I am indebted to Professor H.G. Callan for generous help with experimental work and for invaluable discussion, to Mrs. L. Lloyd for her patient technical assistance, especially during the preliminary stages of my research, to Professor R.M. Cormack for advice on the statistical treatment of the initiation interval correlation in Table 2, to Dr. C. Bostock, Dr. D. Malcolm and Dr. J. Sommerville for helpful and fruitful discussions and to my husband, Dr. I.D. McFarlane for his patient reading of the text of this thesis. Above all, I would like to express sincere thanks to my supervisor, Professor H.G. Callan for his interest, encouragement and guidance in all stages of this research.

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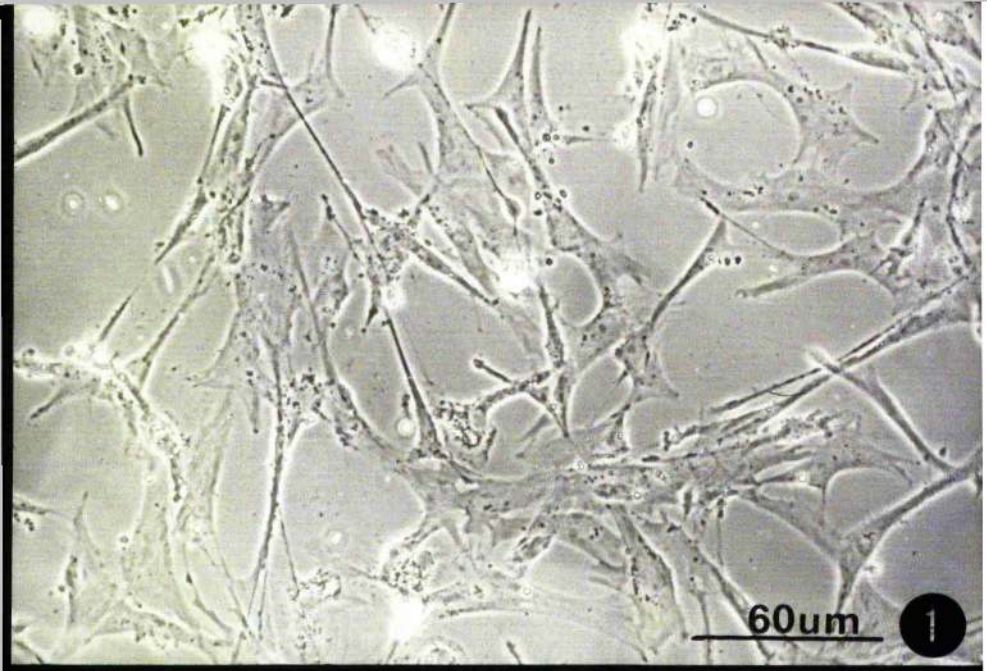
Plate 1. Chick somatic cell monolayer grown in tissue culture at 37°C. Coverslips were laid down in flasks and cell monolayers allowed to form over them. These coverslips were removed from the flasks, mounted on slides and photographed under phase contrast.

Plate 2. Chick blastoderm incubated for 18 hours at 37°C, isolated in vitro and immediately photographed.

p. primitive streak; a.p. area pellucida; a.o. area opaqua. The diameter of the blastoderm is about 8 mm.

Plate 3. A metaphase chromosome complement from chick somatic cells in culture, fixed directly on a slide in 3 parts absolute methanol to 1 part glacial acetic acid, air-dried and stained in 2% aceto-orcein. 6 pairs of large chromosomes are apparent. The estimated number of microchromosomes in the diploid chick cell is 68 (Hammer, 1970).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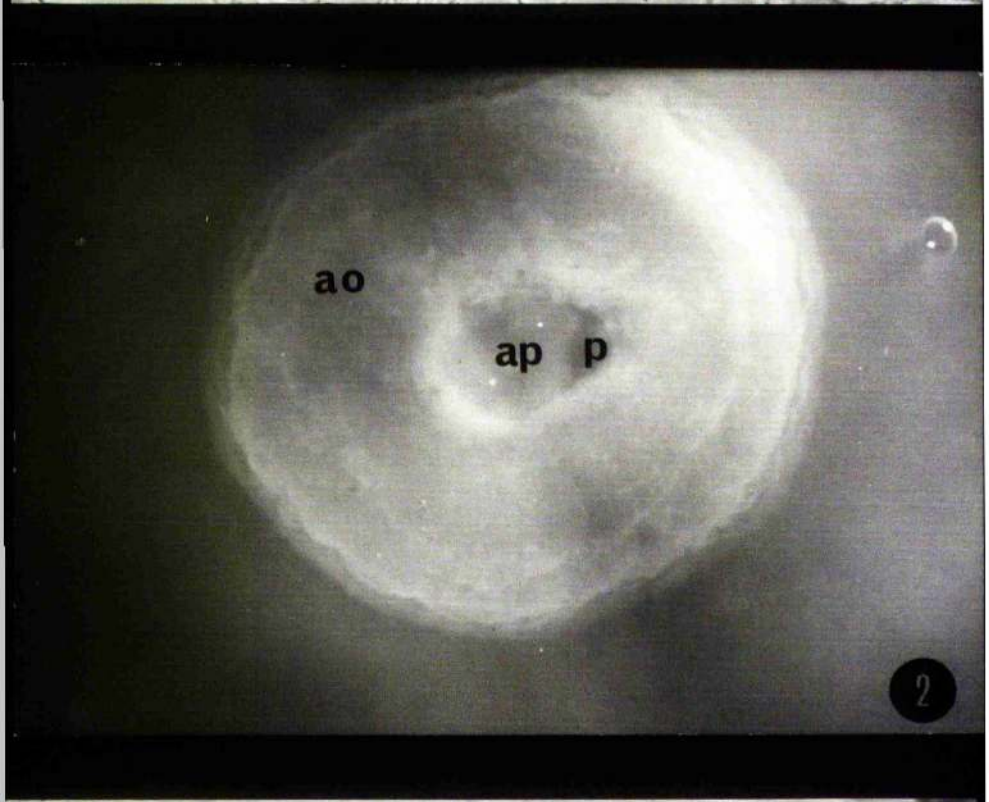
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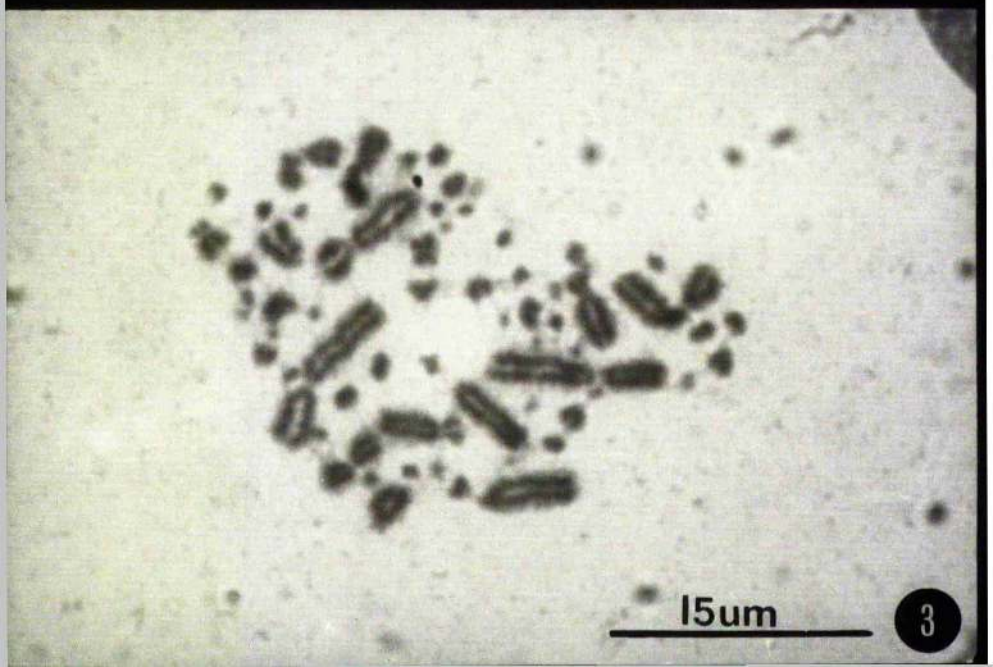
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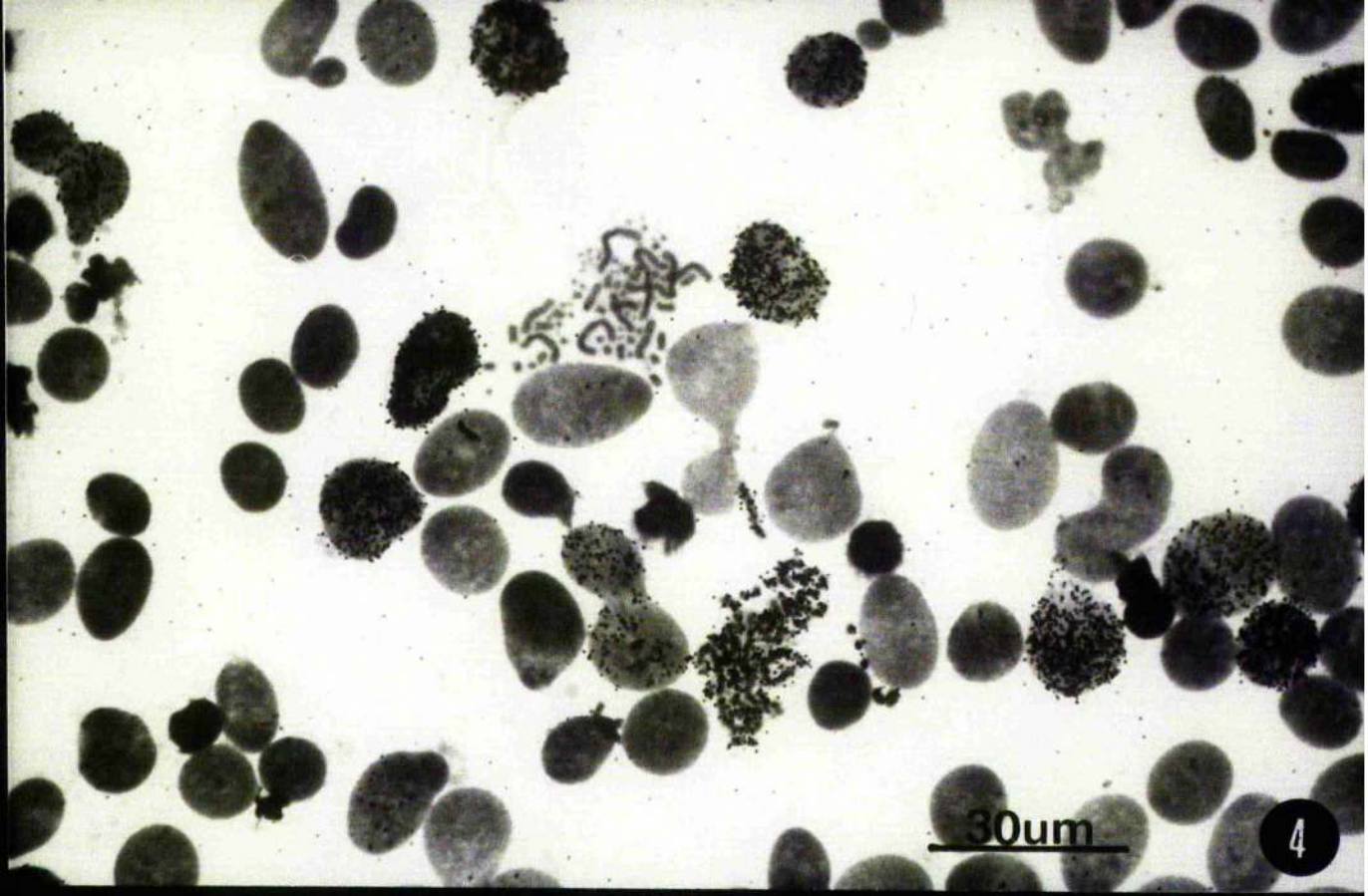
Plate 4. Representative portion of a whole cell autoradiograph used in the experiment to determine the S-phase duration in chick somatic cells in culture at 37°C. Cells were pulse-labelled with ^{3}H-TdR and then allowed to continue growth in unlabelled medium for up to 22 hr. This autoradiograph was prepared from cells harvested 8 hr after the termination of labelling. Both a labelled and an unlabelled set of metaphase chromosomes are shown. Labelled mitoses and interphases are of cells which were in S when ^{3}H-TdR was applied. Unlabelled mitoses and interphase cells represent cells in G_1, G_2 or M when ^{3}H-TdR was applied.

Plate 5. Representative portion of a whole cell autoradiograph used in the experiment to determine the duration of $G_1 + G_2 + M$ in the 12 to 27 hr-incubated chick blastoderm. This example is from a preparation of cells harvested following continuous labelling with ^{3}H-TdR for 12 hr. Notice the 4 unlabelled nuclei at lower centre.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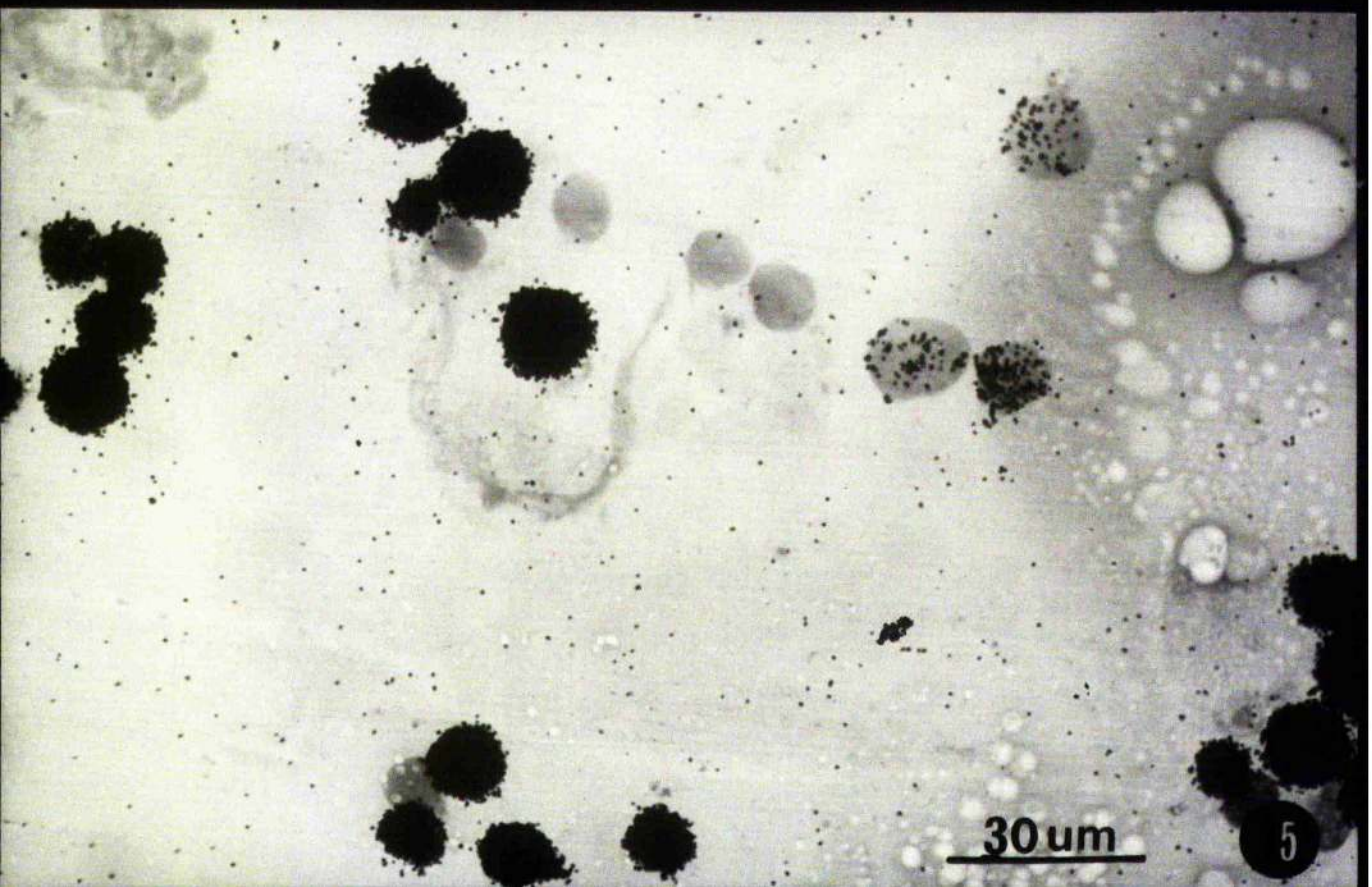
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Plate 6. The effect of the addition of 4×10^{-6} M FUdR and uridine on chick somatic cells in culture. Control cells were untreated, while experimental cells were treated with FUdR and uridine for 16 hr. Both sets of cells were then pulse-labelled with ^{3}H-TdR and whole cell autoradiographs prepared.

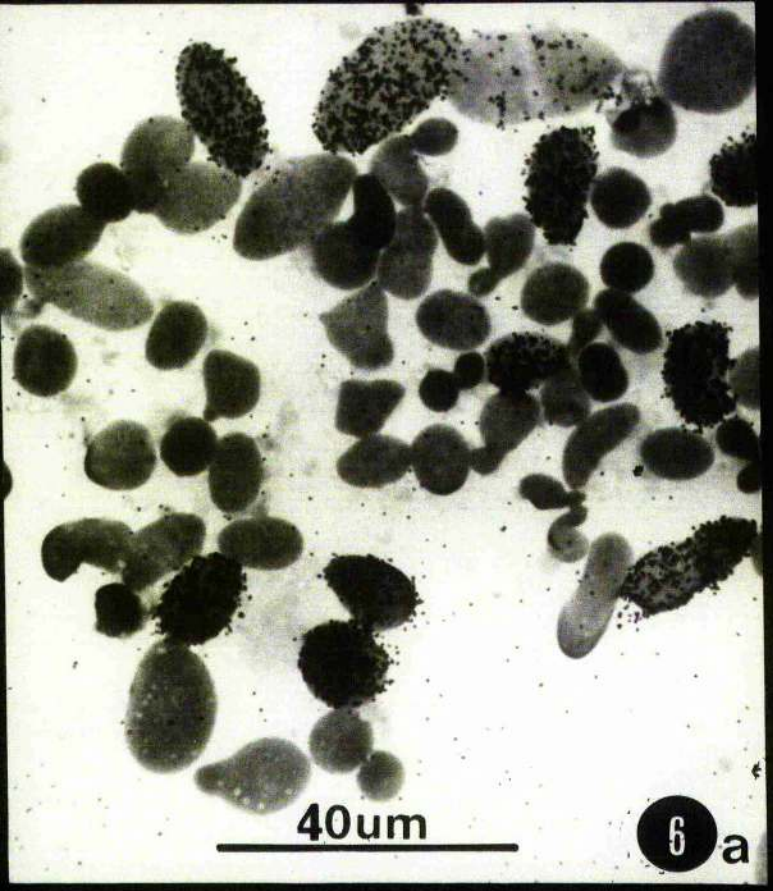
(a) whole cell autoradiograph from control cells

(b) whole cell autoradiograph from experimental cells. The observed increase in the number of labelled cells following FUdR treatment represents the fraction of cells synchronized at the beginning of S by FUdR.

Plate 7. Two DNA fibre autoradiographic preparations photographed under dark-field conditions. Each photograph shows the silver grain distribution in the over-lying autoradiographic film from a millipore filter derived from a dialysis chamber in which DNA was isolated from labelled chick somatic cells.

(a) DNA fibre autoradiograph with silver grain distribution indicative of too high a density of DNA fibres on the under-lying millipore filter.

(b) DNA fibre autoradiograph with a sparse but sufficient spread of DNA fibres on the under-lying millipore filter. The diameter of each filter was about 15 mm.



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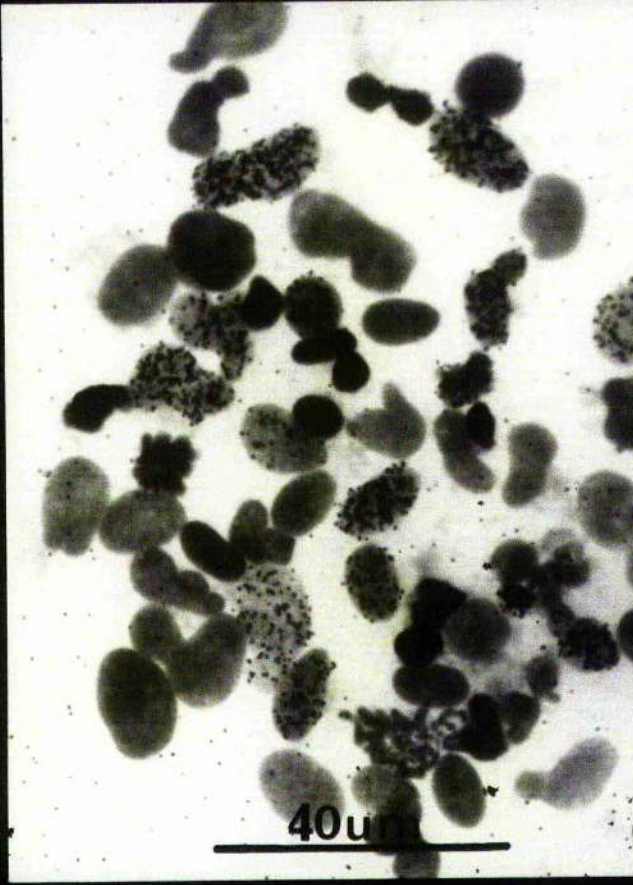

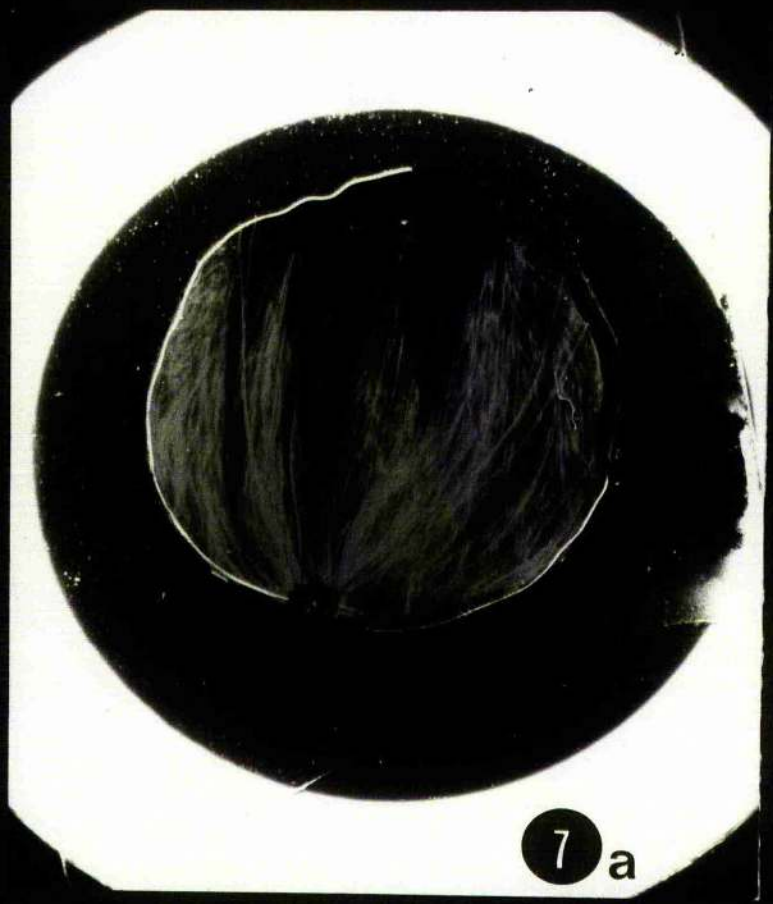

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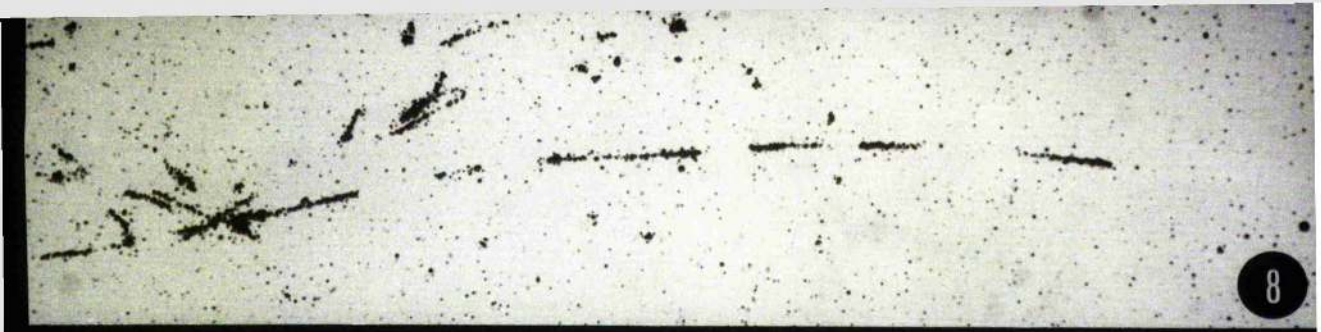
Plates 8 to 22 are photographs of DNA fibre autoradiographs from FUdR-treated chick somatic cells labelled for 30 min and chased for 30 min. Sister strand separation is evident in Plate 11 and in part of the tracks in Plates 10 and 20. Heavily labelled tracks denote regions which were replicated during the first 30 min labelling period, tracks of diminishing grain density (tails) denote regions which replicated during the chase. Heavily labelled tracks with tails at both ends represent regions which initiated during the labelling period, whereas heavily labelled tracks with tails at one end only represent continuing replication from regions which had already initiated before labelling. Both of these types of tracks are mostly to be seen coming into confluence with their neighbours. The heavily labelled track carrying a tail, second from the right in Plate 8 faces at its abrupt end a converging tail from a neighbouring replication unit; such a unit may be considered to be an example of unidirectional replication. All other units in Plates 8 - 22 show grain distribution patterns in accordance with bidirectional replication. The clustering of concurrently active tandem replication units in Plates 8 - 22 suggests that units initiating more or less simultaneously often tend to be clumped together in small groups. The series of tracks in the centre of Plates 16 and 17 provide the kind of evidence which negates the existence of defined termini. Further explanation in text.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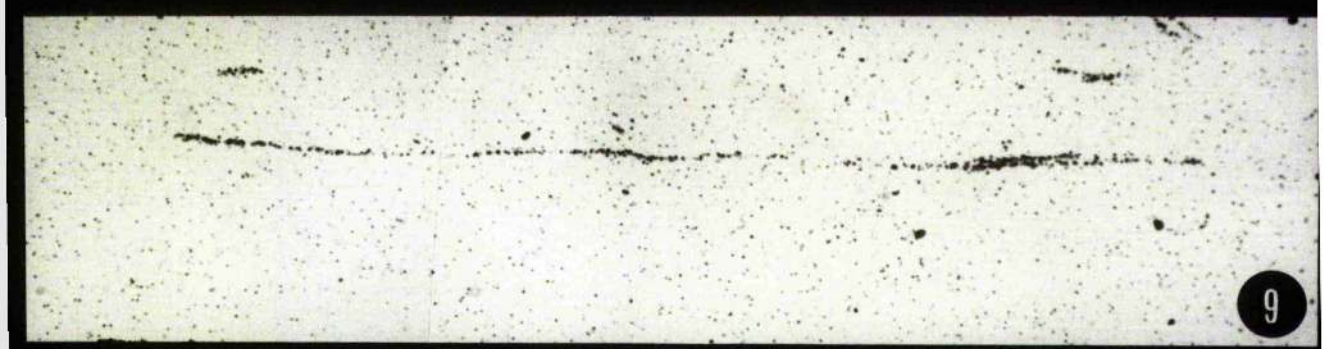
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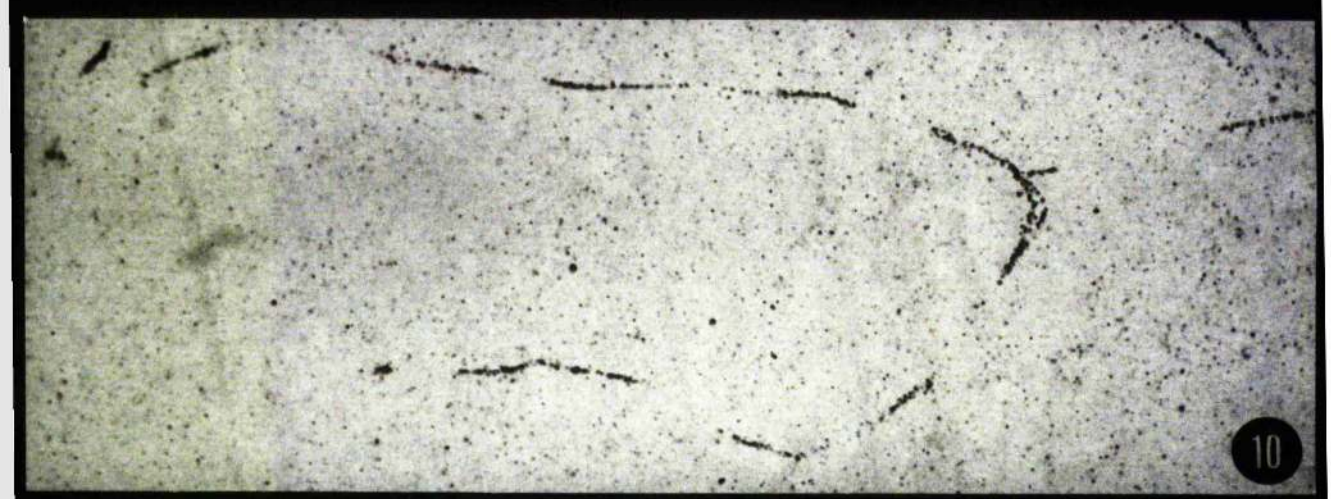
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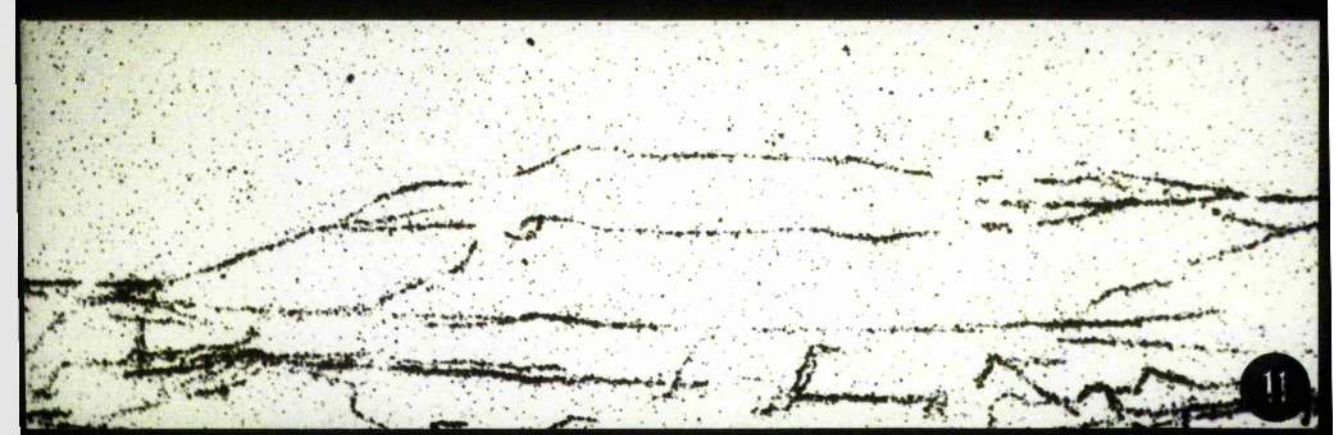
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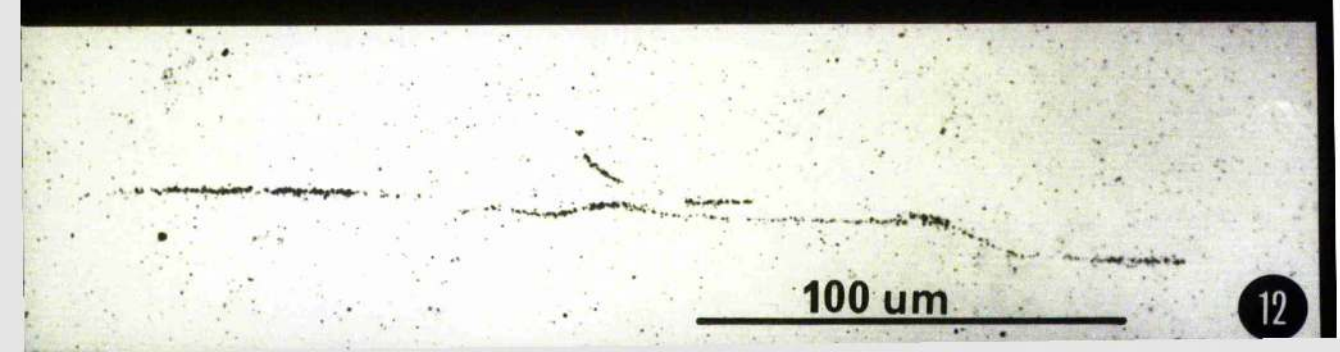
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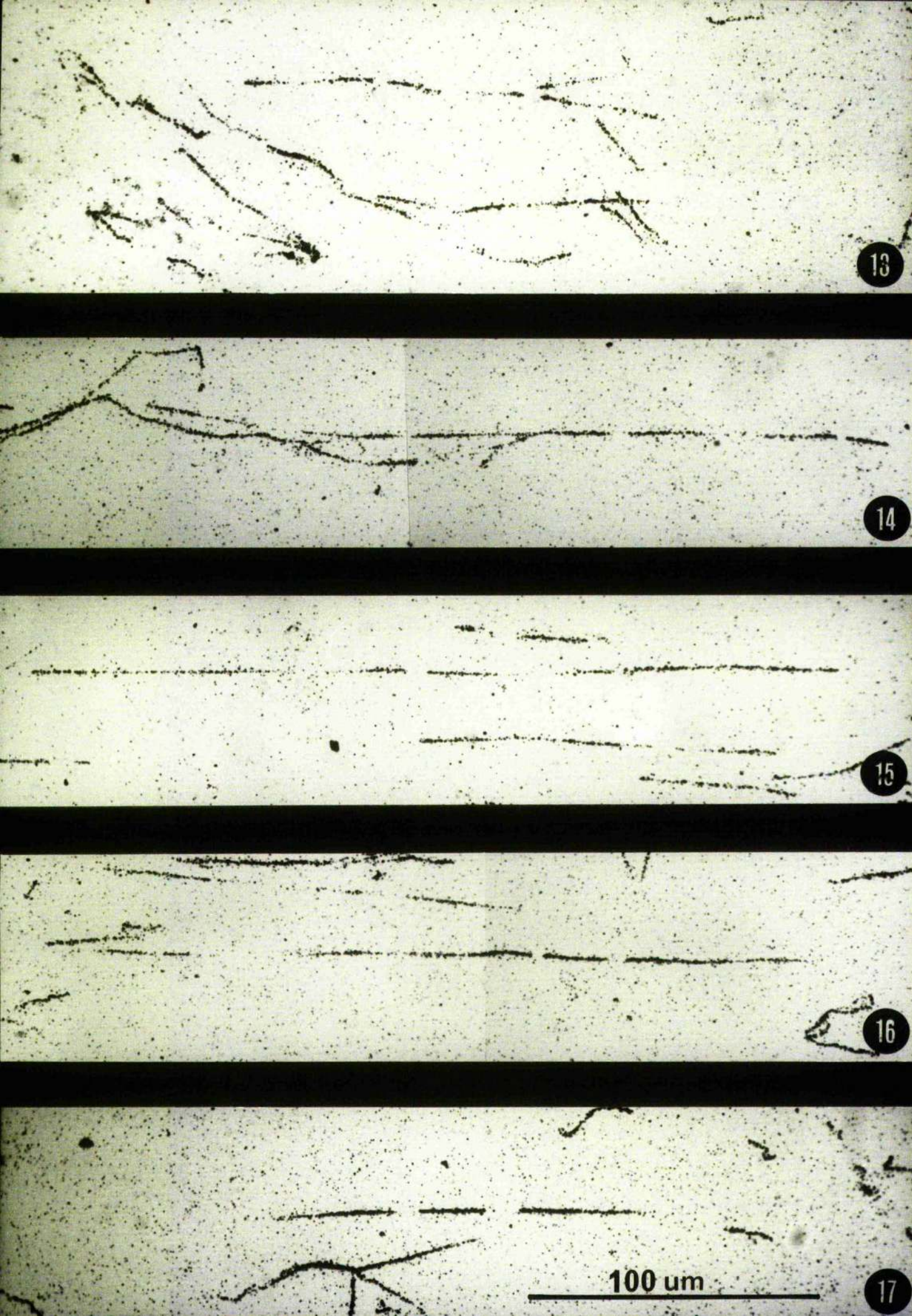
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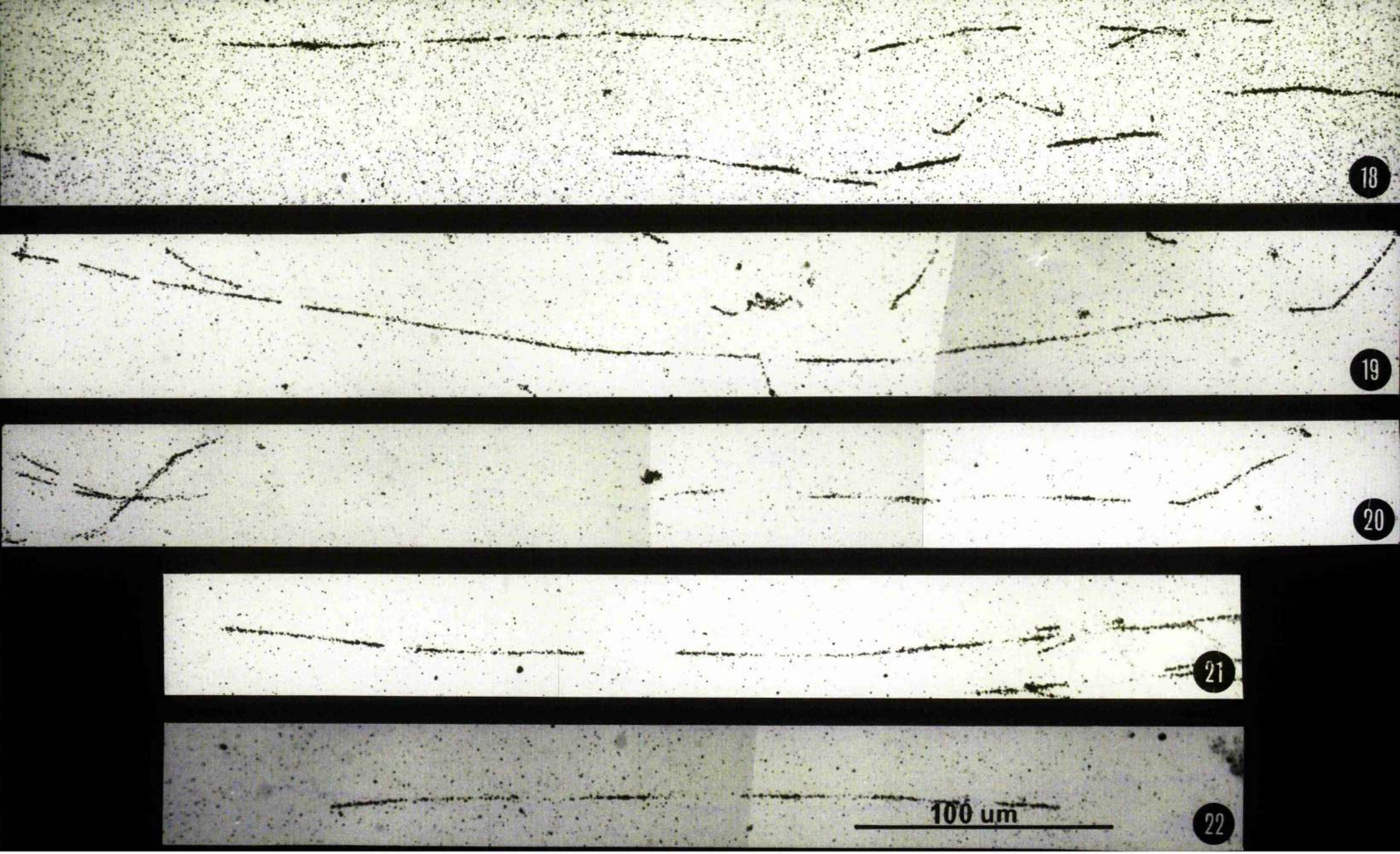
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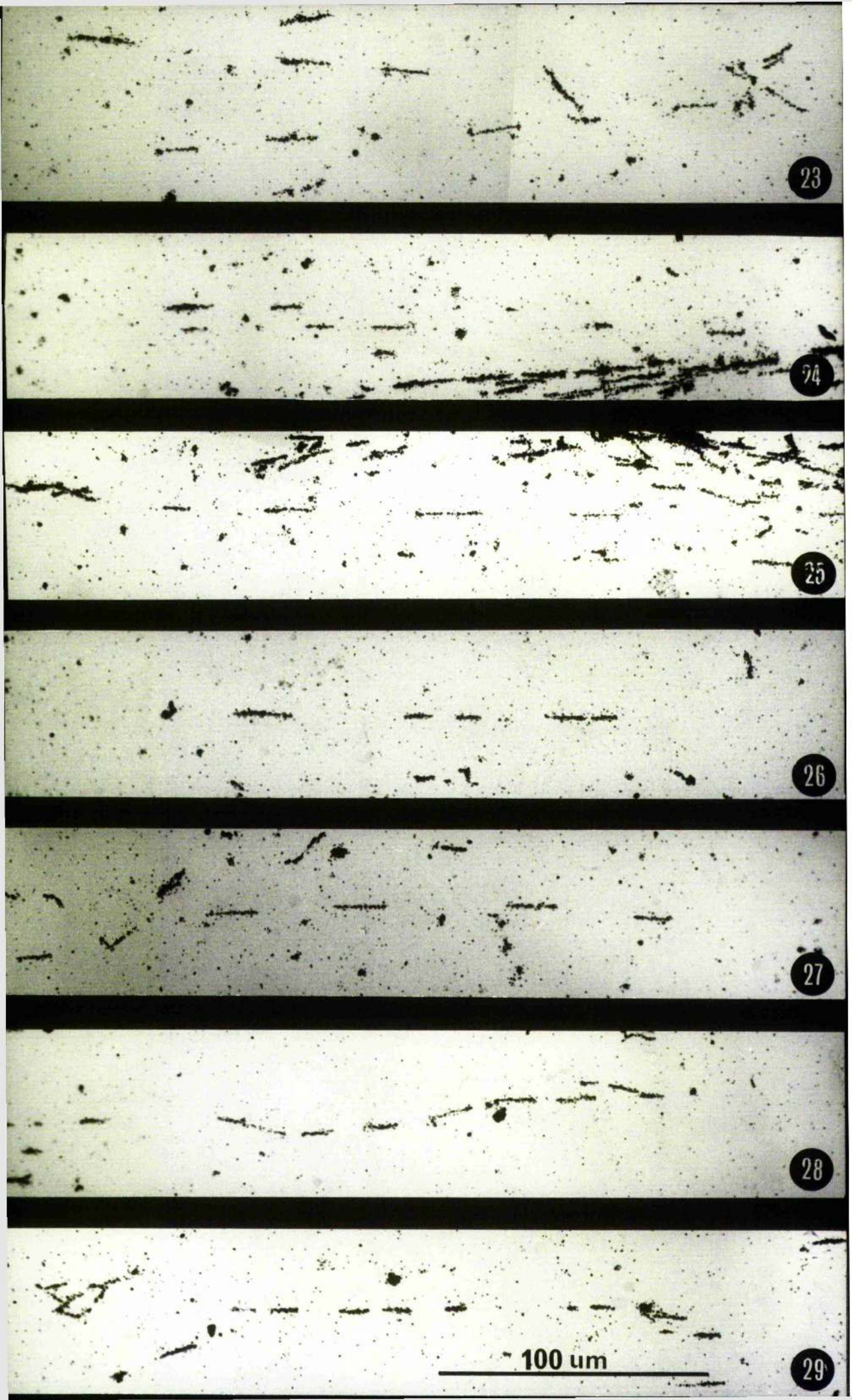
Plates 23 to 33 are photographs of DNA fibre autoradiographs from FUdR-treated chick somatic cells labelled with ^{3}H-TdR for 15 min. Sister strand separation is rarely evident after 15 min because continuous long lengths of DNA have not been replicated in the presence of label. In many cases the concurrently active replication units are clustered in short tandem arrays, bordered at either side by unlabelled, presumably unreplicated DNA, as in Plates 26, 28 and most notably in Plate 32.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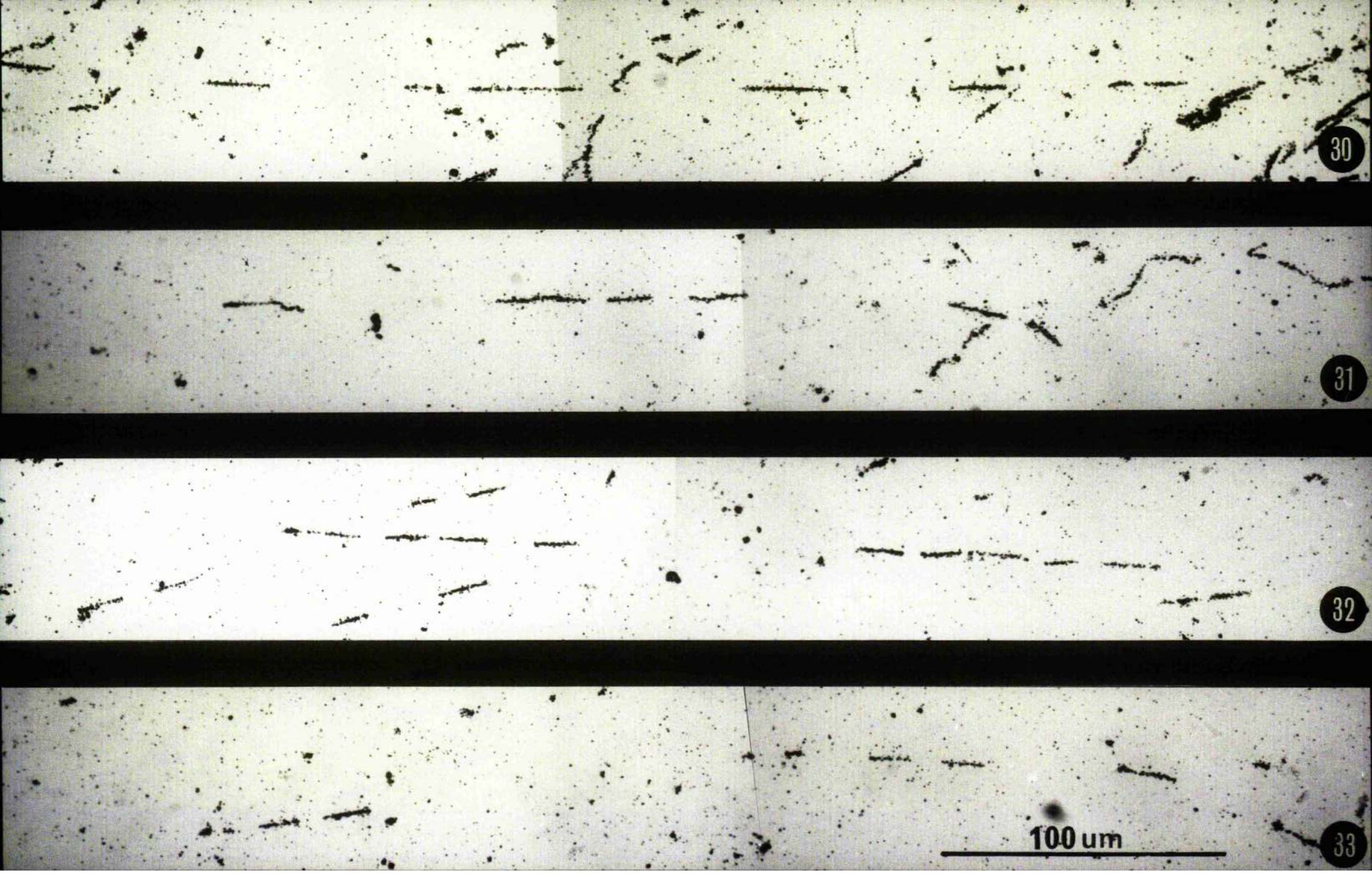
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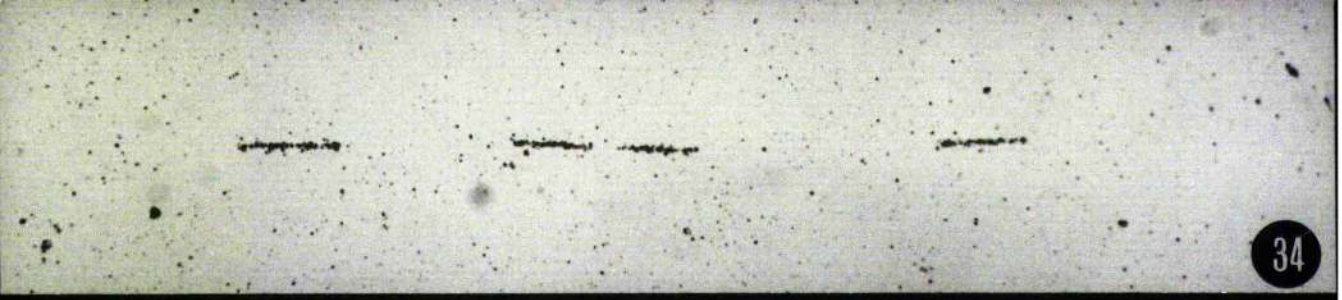
Plates 34 to 43 are photographs of DNA fibre autoradiographs from FUdR-treated chick somatic cells labelled with ^{3}H-TdR for 30 min. Plate 39 shows sister strand separation. As in other DNA fibre autoradiographs shown in this study, concurrently active replication units often occur close together in tandem sequence, e.g. Plates 38 and 39.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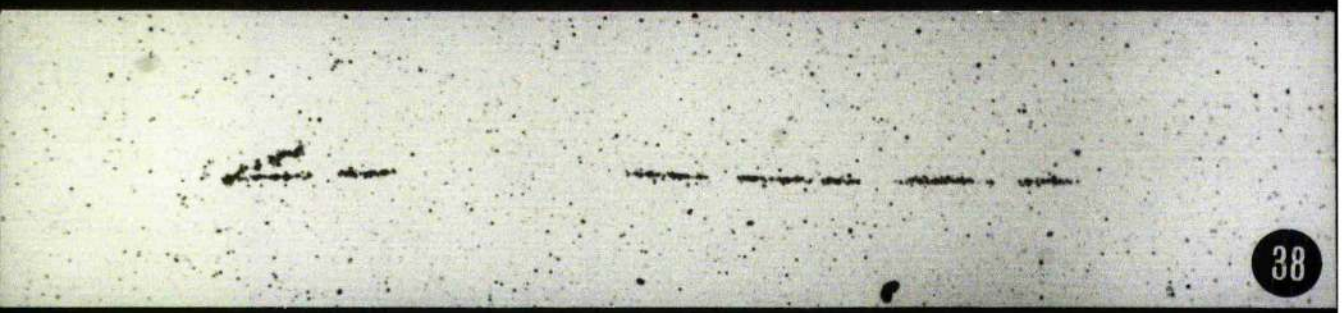
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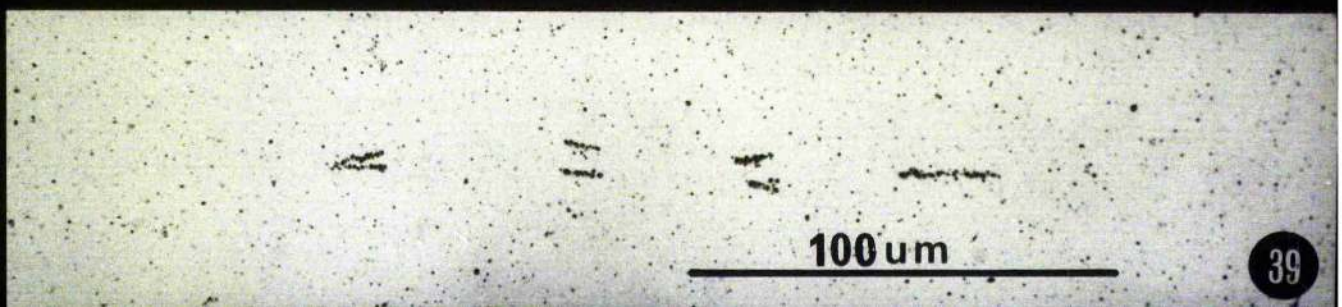
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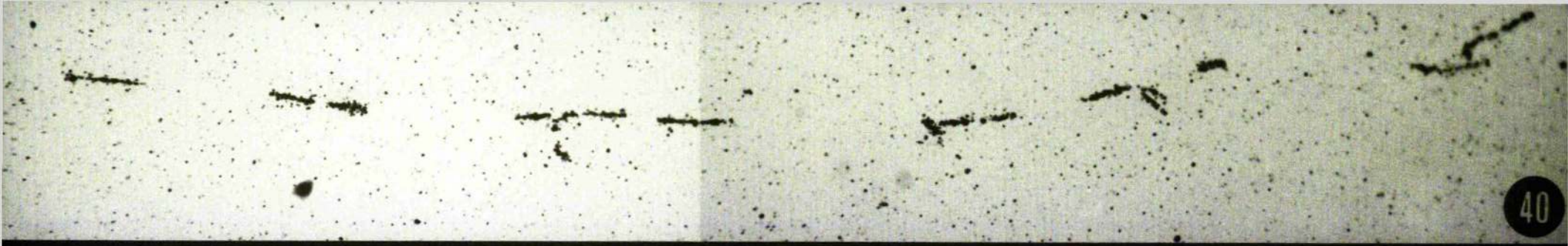
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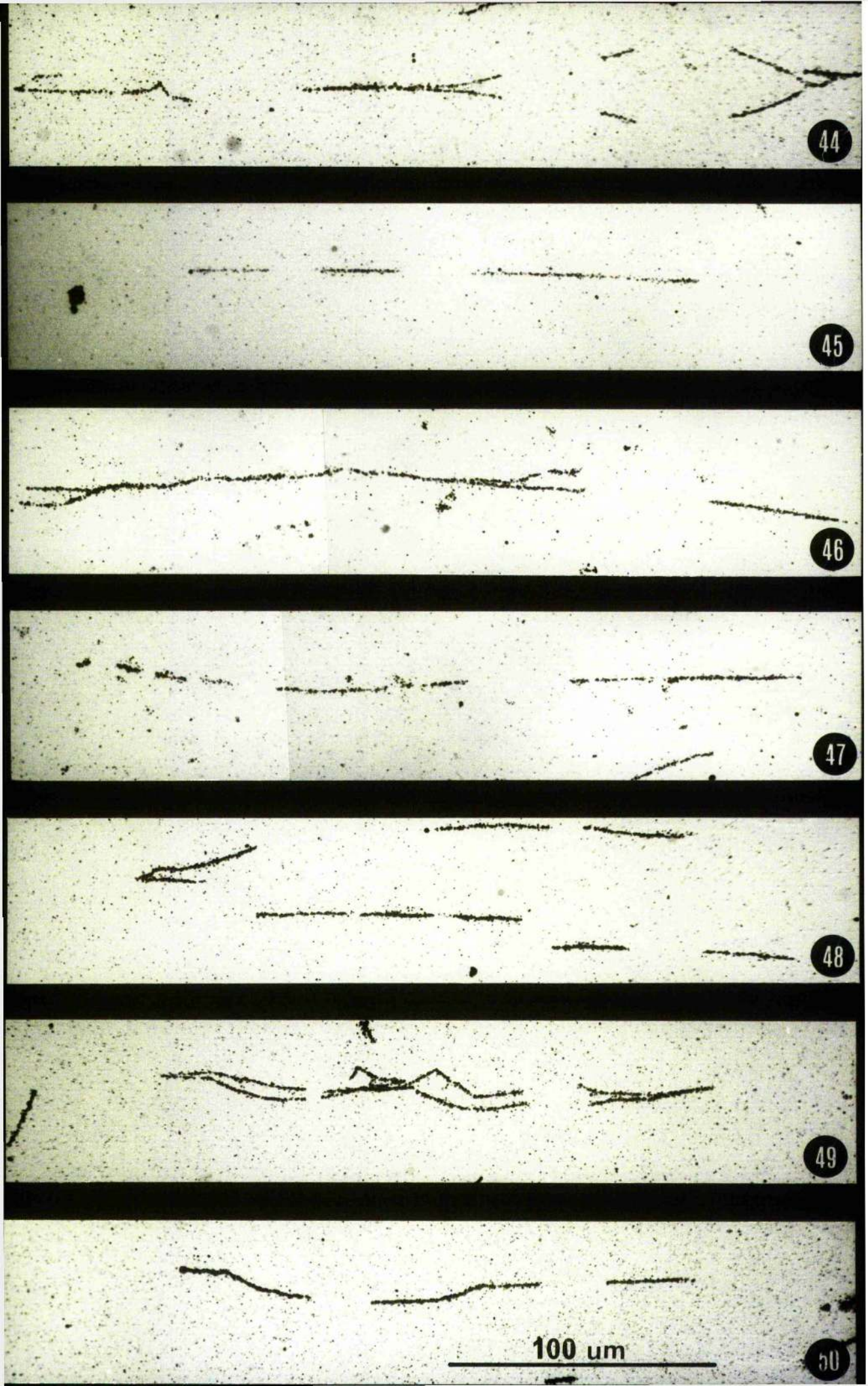
Plates 44 to 56 are photographs of DNA fibre autoradiographs from FUdR-treated chick somatic cells labelled with ^{3}H-TdR for 60 min. Many examples of sister strand separation are evident where long continuous lengths of DNA have replicated in the presence of ^{3}H-TdR and the newly synthesized strands have moved apart, e.g. Plates 44, 46, and 49. Long labelled lengths (Plate 46) which represent the fusion of two or more neighbouring replication units and/or neighbouring one-way sections are more evident after 60 min labelling than in autoradiographs from cells labelled for shorter periods. In Plate 47 the diverse labelled lengths demonstrate that a cluster of replication units having similar characteristics, like the cluster of neighbouring short tracks to the left of the photograph, can occur in tandem with replication units with different characteristics. Another such cluster of short, equal labelled lengths separated by short unlabelled gaps is shown in Plate 56, although in this case the cluster is not in tandem with other dissimilar replication units.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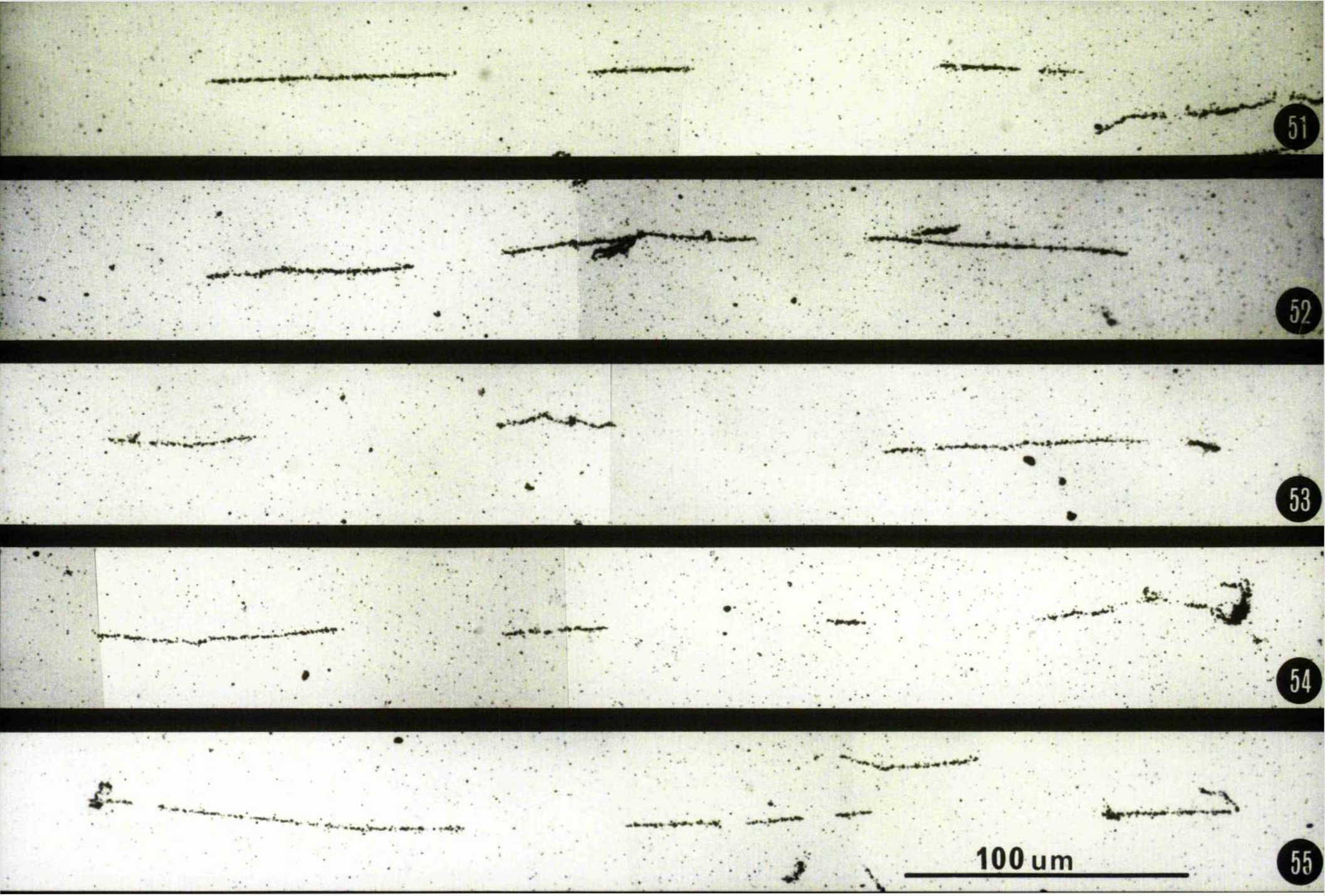
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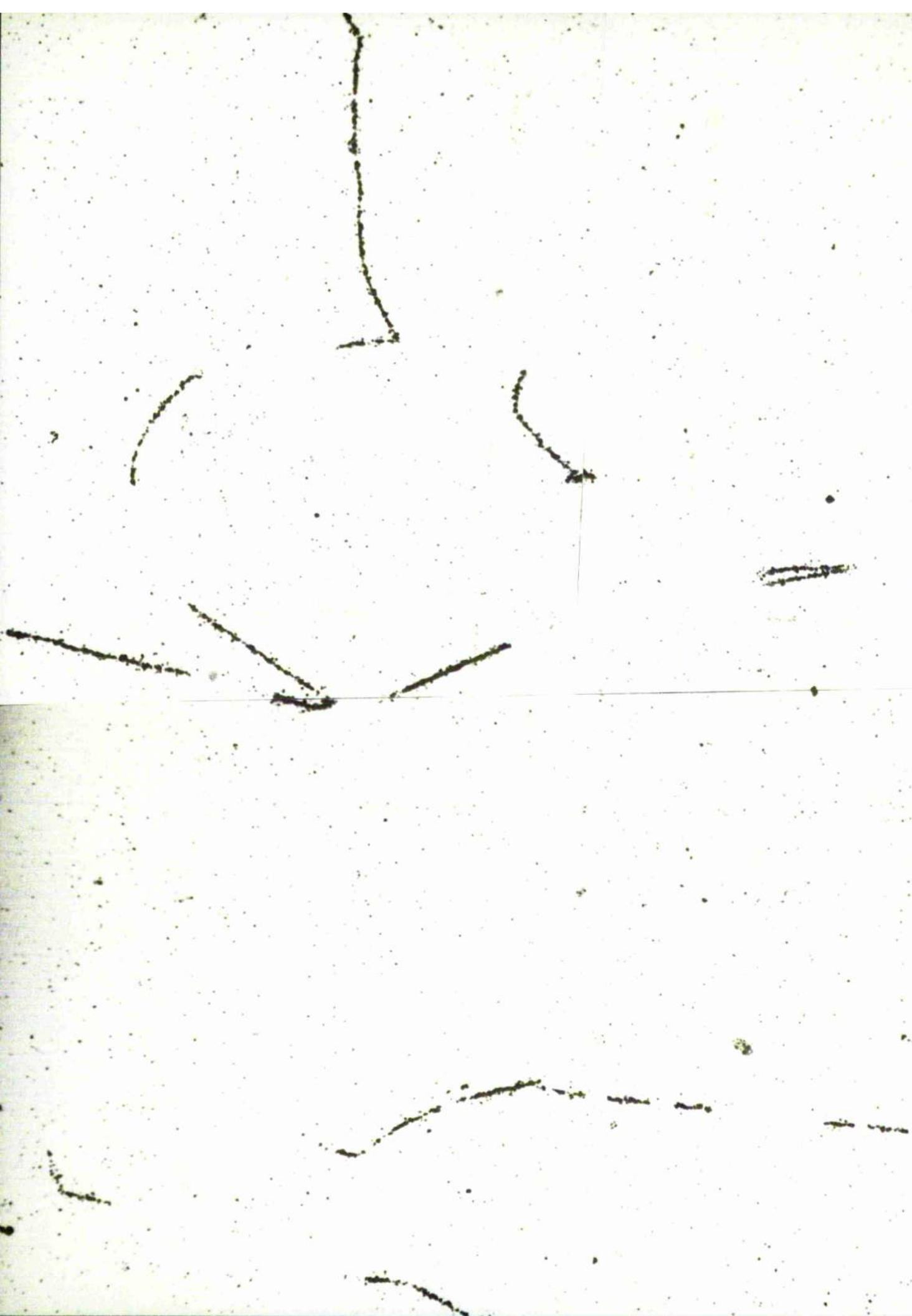
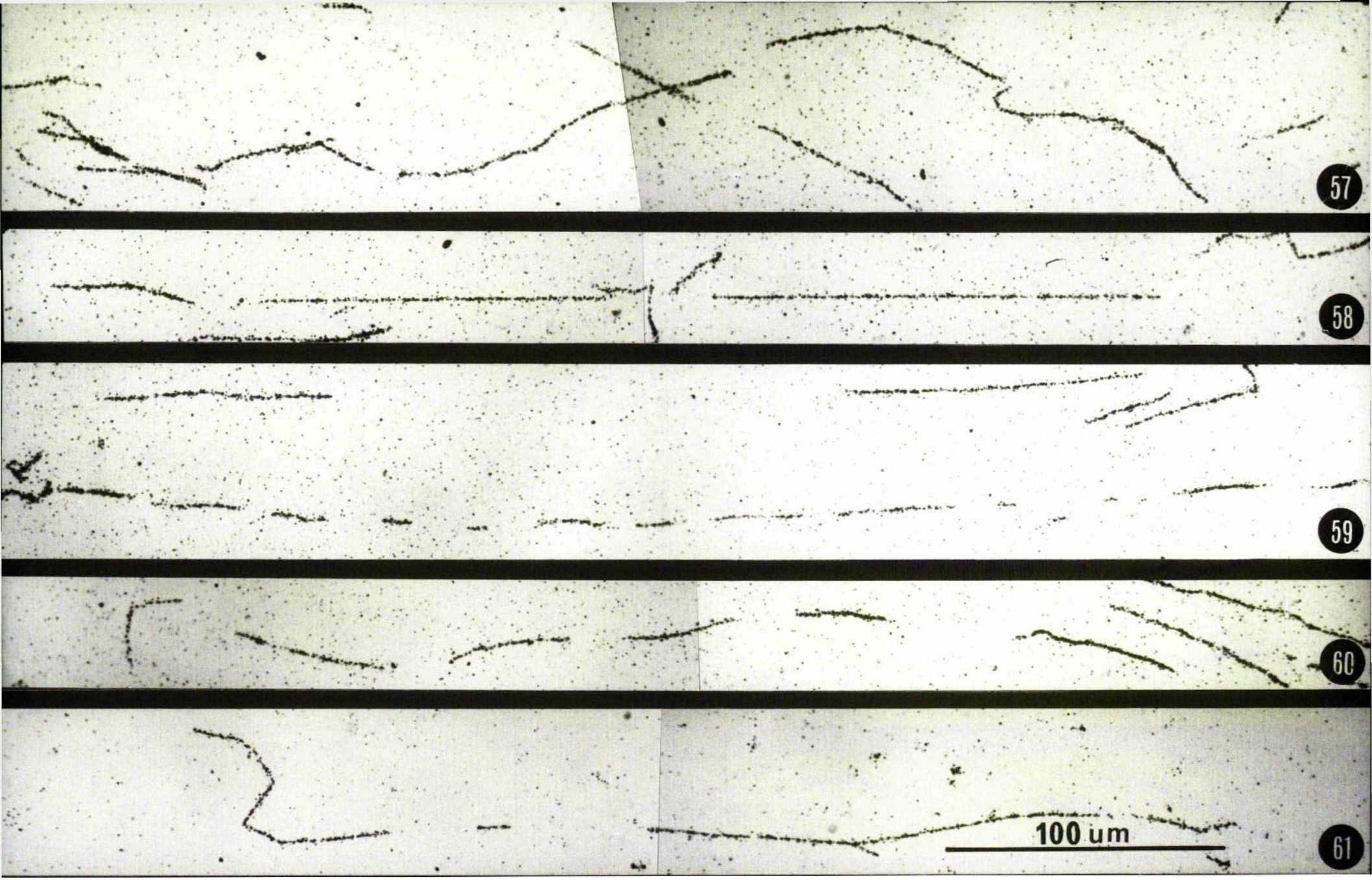
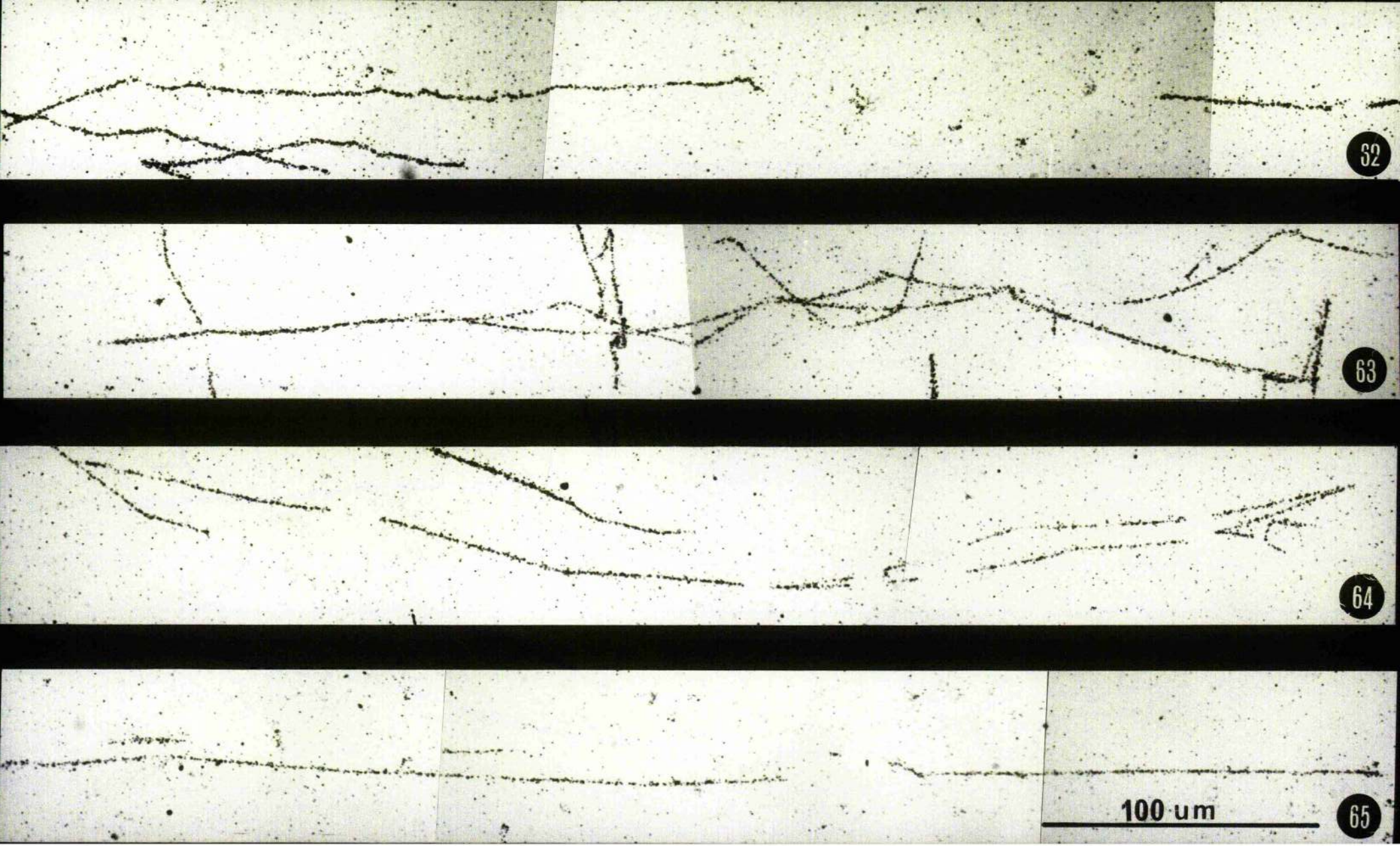


Plates 57 to 65 are photographs of DNA fibre autoradiographs from FUdR-treated chick somatic cells labelled for 120 min. The long tracks are the result of fusions between neighbouring units. Sister strand separation is evident in Plates 63 and 64. The gaps between neighbouring tracks in the split region in Plate 64 denote that replication had already started before provision of label. The long unlabelled gap in the tandem sequence in Plate 62 suggests that this is a portion of the DNA in which replication had not occurred within 2 hr of labelling, i.e. indicating at least 2 hr staggering in initiation times. The long tandem sequence of short similar labelled lengths and short similar non-labelled gaps in Plate 59 demonstrates that neighbouring replication units may have similar times of initiation and similar initiation intervals.


57
58
59
60
61
100 um


62
63
64
100 um
65

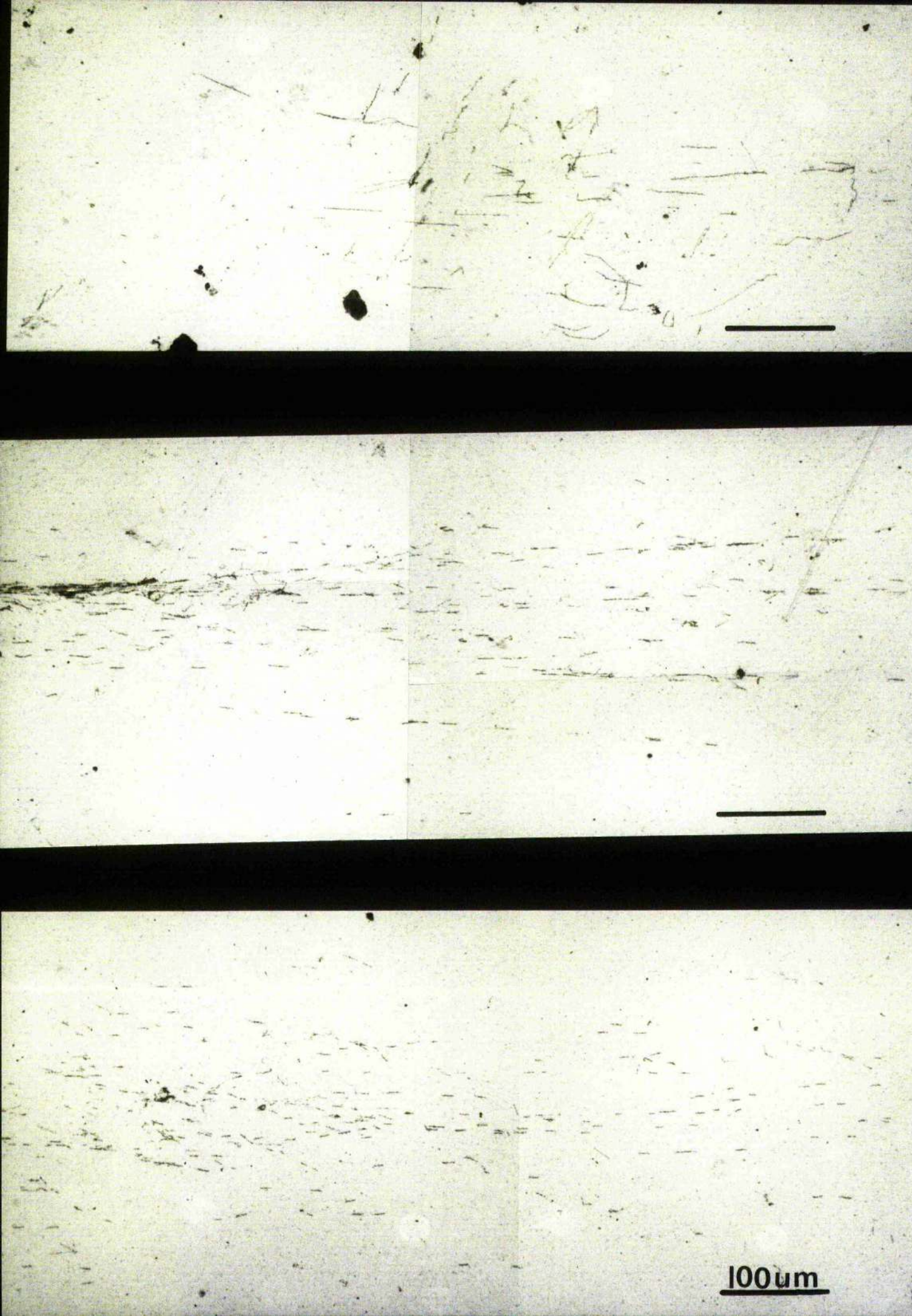
Plates 66 to 85 are photographs of DNA fibre autoradiographs from chick somatic cells not pre-treated with FUdR. Plates 66, 67 and 79 are from cells labelled for 15 min, Plates 68, 69, 70 and 78 are from cells labelled for 30 min, Plates 71, 72, 76 and 77 are from cells labelled for 30 min and chased for 30 min, Plates 74, 75, 80 and 81 are from cells labelled for 60 min and Plates 73 and 82 to 85 are from cells labelled for 120 min. The labelled tracks in these autoradiographs do not always show an increase in length with increasing labelling duration; this is much more obvious in DNA fibre autoradiographs from semi-synchronized cells. Short labelled lengths are often apparent even after 60 and 120 min labelling, as shown in Plates 80, 81, 83 and 85. Such short lengths could arise from fused one-way sections of neighbouring replication units that initiated prior to labelling but which continued replication and fused early in the labelling period or, alternatively they could arise from replication units that initiated near the end of the labelling period. Plate 67, 69, and 70 show examples of small clusters of short labelled tracks of similar length which must arise from initiation sites spaced very close together, far closer than the mean initiation interval. Tails of decreasing grain density reflecting DNA synthesized during the chase period can only be tentatively identified in Plates 71, 72, 76 and 77.

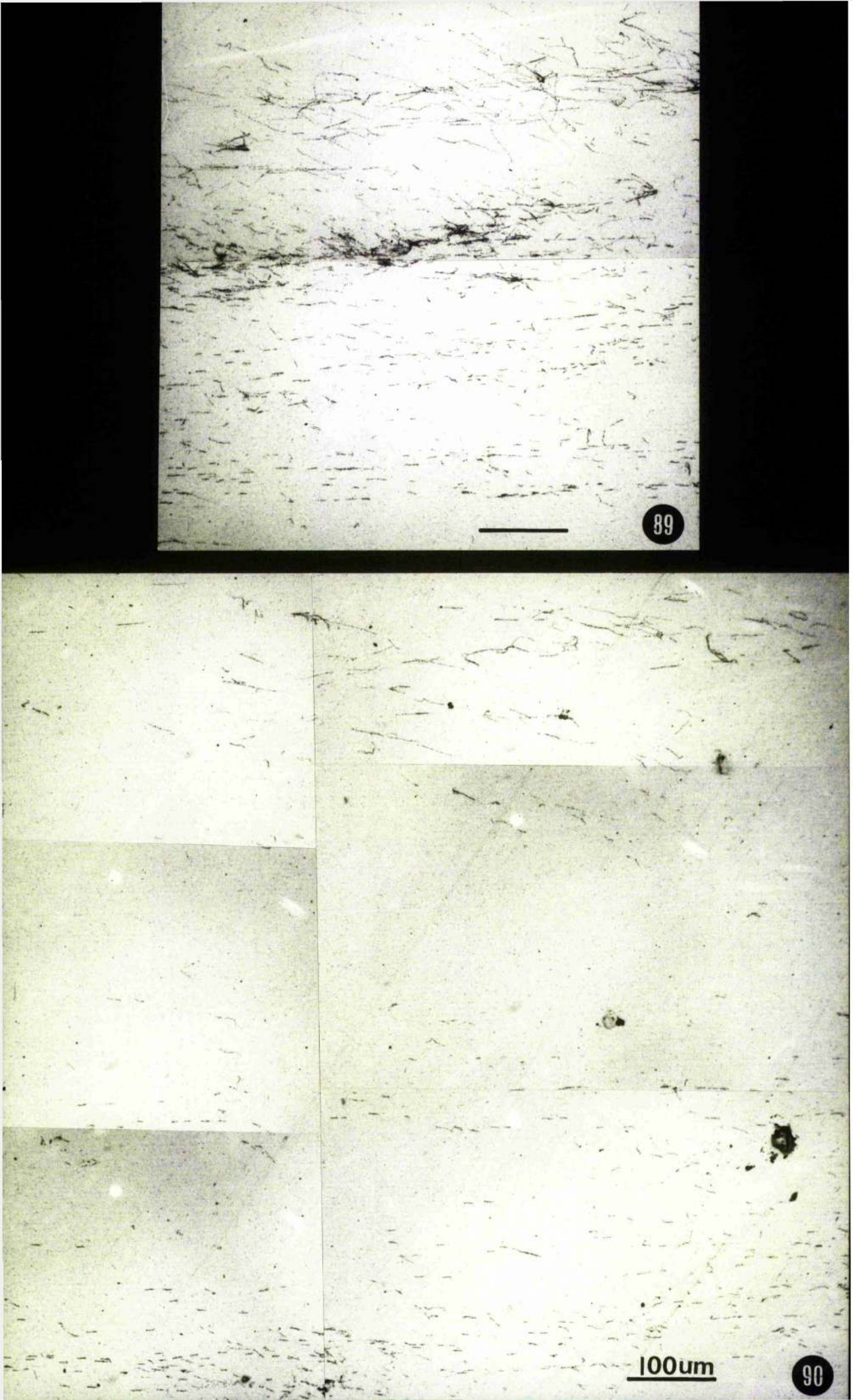

66
67
68
69
70
71
72
100 um
73


74
75
76
77
78
100 um
79


80
81
82
83
84
85
100 µm

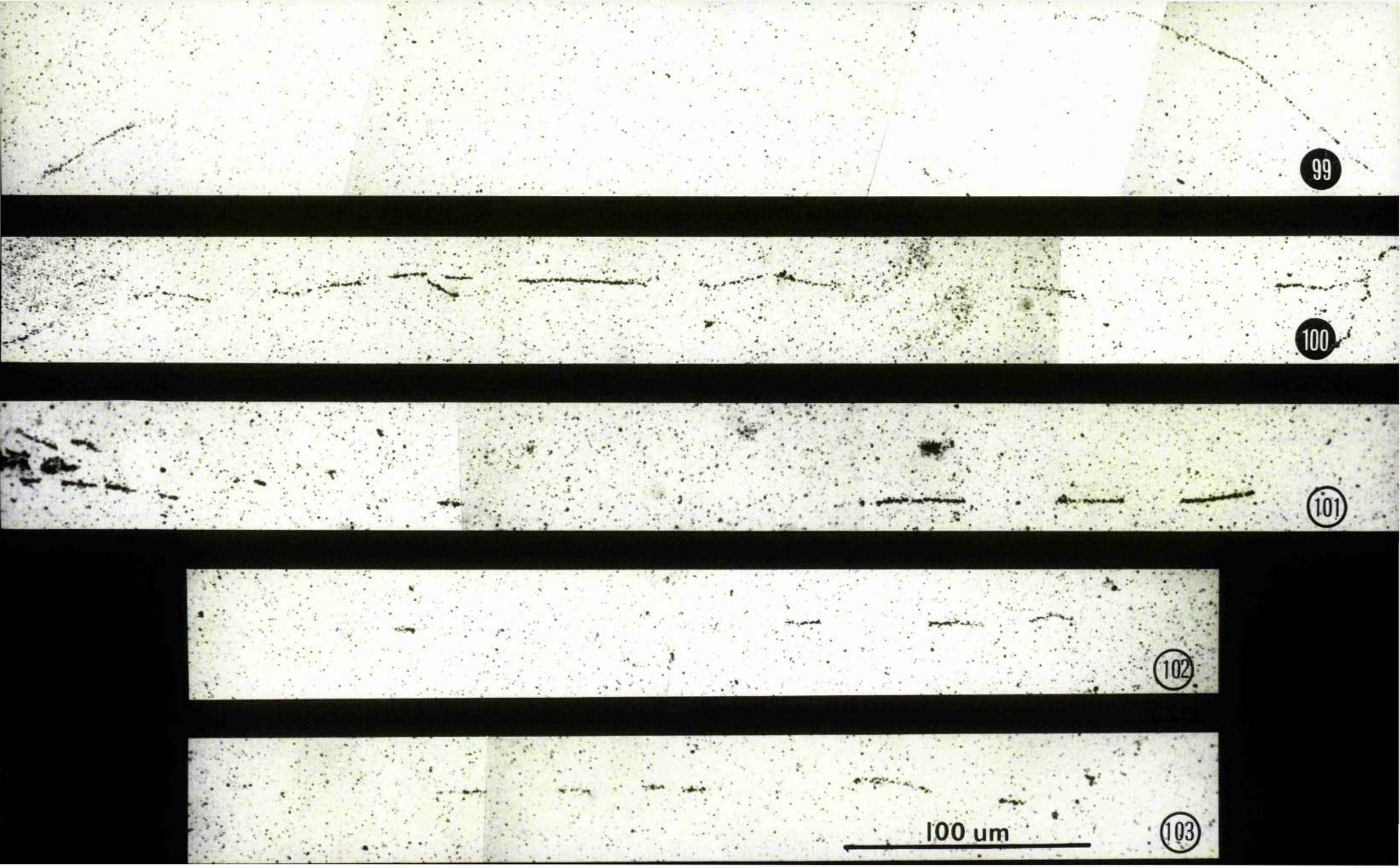
Plates 86 to 90 are low-power photomicrographs of DNA fibre autoradiographs from chick somatic cells not pre-treated with FUdR and all labelled with ^{3}H-TdR for 60 min. Each photograph shows one or two "sprays" of DNA, the labelled units within each spray are very similar in length. Plate 86 shows a spray containing very long labelled lengths suggesting that fusion may have occurred between closely spaced neighbouring replication units which initiated at a similar time, i.e. some time during the 60 min labelling period. Plate 87 shows a spray containing relatively short labelled units. Plate 88 shows a spray containing very short labelled lengths, far shorter than would be anticipated from the estimated replication rate if the units represented one-way sections. Labelled sections which appear to be in tandem in Plates 87 and 88 are mostly to be seen in clusters, the distance between neighbouring sections being small i.e. less than 60 μm. Plates 89 and 90 each show two adjacent, dissimilar sprays.


100um


89
100um
90

Plates 91 to 107 are photographs of DNA fibre autoradiographs from chick blastoderms incubated for 18 hr isolated *in vitro*, FUdR-treated and labelled with ^{3}H-TdR. Plates 91 and 92 and 101 to 103 are from blastoderms labelled for 30 min; Plates 93 to 95, 99 and 100 and 104 are from blastoderms labelled for 60 min; Plates 96 to 98 and 105 to 107 are from blastoderms labelled for 30 min and chased for 30 min. Sister strand separation is evident in Plate 97 and in part of the tandem sequence in Plate 98. Very long unlabelled lengths separating two labelled units, as in Plates 99, 106 and 107 are more frequently present in these DNA fibre autoradiographs than in those from chick somatic cells. Such long unlabelled lengths suggest that initiation intervals may sometimes be very long and/or that great staggering in the times of initiations occurs. This pattern also demonstrates that DNA replication is not rapidly completed, as is generally the case in embryonic tissues.


91
92
93
94
95
96
97
98
100um


99
100
101
102
103
100 um


104
105
106
~ 100 um
107

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