5 Role of nuclear structure in DNA replication

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1. Introduction

The idea that DNA polymerases track along the template as they duplicate the DNA pervades our thinking (Figs 1a–c) (1, 2; and this volume). We imagine that it must be the small polymerase that moves along the very much larger template and that each polymerase acts relatively independently of others. However, recent evidence suggests many active polymerases are concentrated together into large nuclear structures – 'factories' – and that replication occurs as a number of templates slide past the many enzymes fixed in the factory; the template moves whilst the

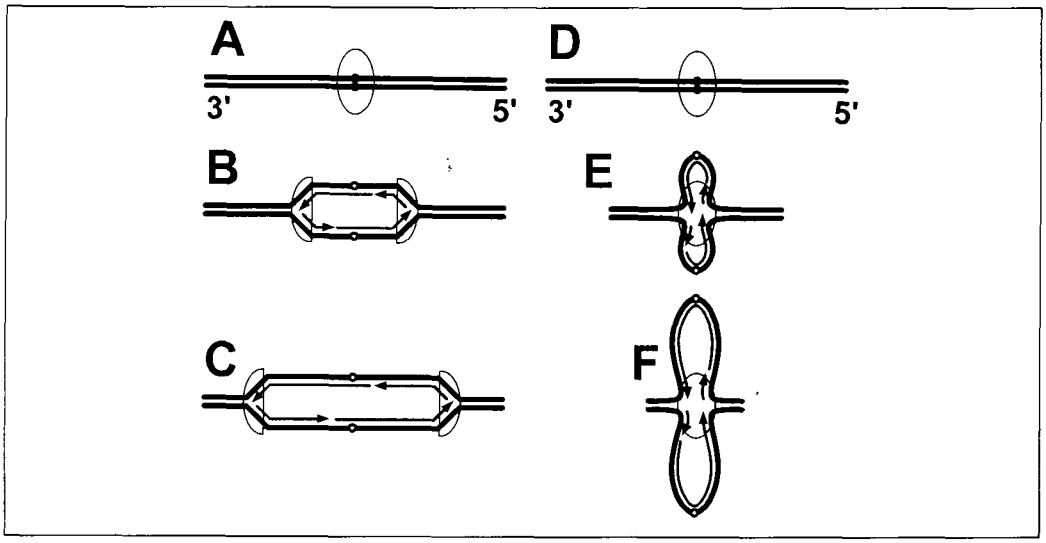

Fig. 1 Models for replication involving mobile (a–c) and immobile (d–f) polymerases. (a–c). The conventional model involves (a) binding of the polymerizing complex (oval) to the origin (black circles), before the complex splits into two; (b, c) the two halves then track along the template (thick lines) as nascent DNA (thin grey lines) is made. Arrows indicate direction of growth of nascent chains. (d–f) The alternative model involves passage of the template through a fixed complex and extrusion of nascent DNA. The origin is shown here detaching from the complex, but it may remain attached. Redrawn from Hozák and Cook (79).

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enzyme remains stationary, rather than vice versa (Figs 1d–f). Then, higher-order structure dictates how and when DNA is replicated. Discussion concentrates on eukaryotic enzymes, but as the catalytic domains of all polymerases are structurally related (3), prokaryotic enzymes probably work in the same way.

Jacob *et al.* (4) first suggested in their 'replicon' model that polymerases might be attached to a larger structure; attachment of the bacterial chromosome to the membrane would regulate initiation of replication and specific growth of the membrane between the two attached progeny chromosomes could ensure that they segregated correctly to daughter cells. Although membranes do play several roles in these processes, they are not those that were initially envisaged (5, 6). Nevertheless, this model provoked a search for analogous membrane attachments in eukaryotes and this eventually led to the discovery that nascent DNA was attached, not to the membrane, but to various subnuclear structures, including 'matrices' and 'cages'. (For reviews, see 7–9.) As nascent DNA is sticky and as polymerases sometimes aggregate during extraction in the unphysiological salt concentrations used during isolation (10), it was easy to dismiss these results as reflecting nothing more than the artefactual precipitation of soluble polymerases on to an underlying structure (11). And as, at about this time, protein chemists were isolating relatively pure polymerases and finding that they worked *in vitro* in the absence of any larger structures, they included no such structures in their models. And, of course, it is these models that fill our textbooks.

2. Artefacts

Isotonic salt concentrations were not initially used during fractionation, or during polymerase assay, because they cause chromatin to aggregate into an unworkable mess. Therefore biochemists used more tractable conditions, often isolating nuclei and chromatin in (at least) one-tenth the physiological salt concentration. But this destroys the 30 nm chromatin fibre, extracts most DNA polymerases, and doubles the number of attachments of the chromatin fibre to the substructure. Often residual aggregation is suppressed by adding 'stabilizing' cations but these generate further artefactual attachments. Therefore it is not surprising that the slightly different conditions used to isolate 'matrices', 'scaffolds', and 'cages' ensures that each has its own characteristic set of sequences associated with a different subset of proteins. For example, matrix-attached regions or MARs are bound to various different proteins depending on the precise method of isolation, scaffold-attached regions or SARs are often specifically associated with topoisomerase II, and transcribed sequences are bound to 'cages'. Sceptics point to these differences and naturally suggest that some, or all, of these various complexes are artefacts and have no counterparts *in vivo* (11, 12).

Against this background, it was not surprising that convincing evidence for a role of larger structures in replication was only obtained when more physiological conditions were used.

3. Attached polymerases

The practical problems caused by the aggregation of chromatin at isotonic salt concentrations can be overcome if cells are encapsulated in agarose microbeads (50–150 μm diameter) before lysis. As agarose is permeable to small molecules such as nutrients, encapsulated cells grow in standard tissue culture media. They can also be permeabilized with a mild detergent in a 'physiological' buffer (13); then, most soluble cytoplasmic proteins and RNA diffuse out to leave the cytoskeleton surrounding the nucleus (Figs 2a–c). These cell remnants are protected by the agarose coat; importantly, they can be manipulated freely without aggregation whilst remaining accessible to probes like antibodies and enzymes. While no isolate is free of all artefacts, it seems unlikely that polymerases could have aggregated artefactually in this one: template integrity is retained, essentially all replicative activity of the living cell is preserved, and this activity increases 50-fold from low levels as cells pass from G1 into S phase.

Models involving tracking or immobile (i.e., attached) polymerases can be distinguished by cutting this encapsulated chromatin into fragments of <10 kb with an

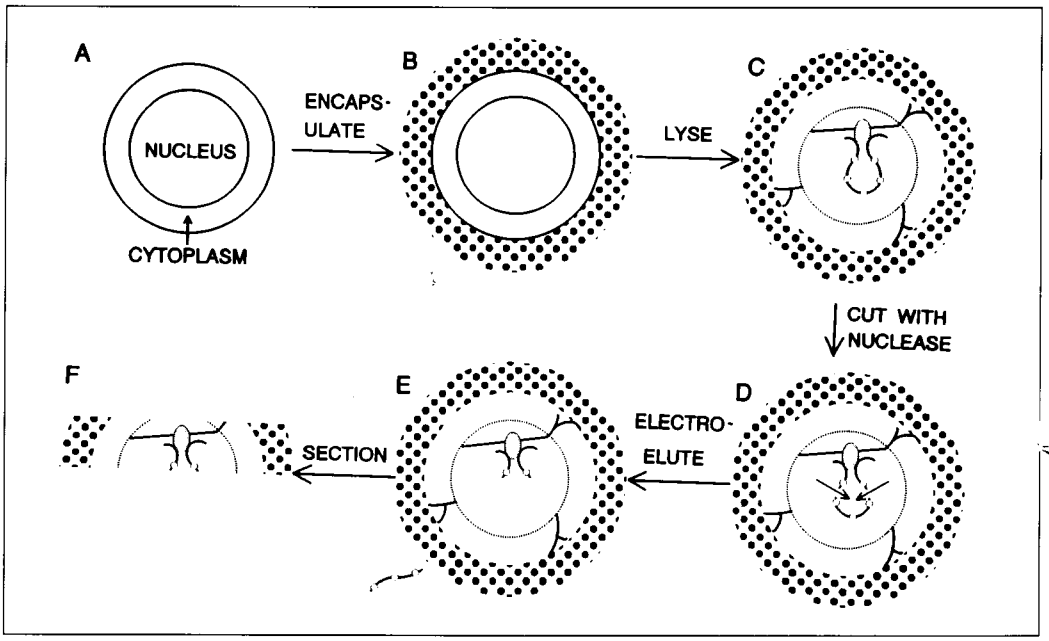


Fig. 2 Procedure for visualizing nucleoskeletons and associated structures. Cells (a) are encapsulated in agarose (dots) (b) and lysed (c) to leave a cytoskeleton, nuclear lamina (dotted circle), and nucleoskeleton (straight line) to which is attached a replication 'factory' (grey oval) and a DNA loop covered with nucleosomes (open circles). Dense chromatin obscures the nucleoskeleton but can be removed by (d) cutting the chromatin fibre with a restriction enzyme, and then (e) removing any unattached fragments electrophoretically. (f) After cutting thick sections, the nucleoskeleton and associated factory can now be seen in the electron microscope. From Cook (76) with permission of ICSU Press.

endonuclease and then removing electrophoretically any unattached fragments (Fig. 2). If polymerizing complexes are attached to a large structure (e.g., the grey oval that is attached in turn to the skeleton in Fig. 2c), they should remain in the agarose bead after electroelution; if unattached, they should electroelute from the bead with the eluting chromatin fragment in Fig. 2e. Cutting chromatin in unsynchronized and permeabilized HeLa cells, followed by electroelution of 75% of the chromatin, hardly reduced DNA polymerizing activity. As very large chromatin fragments (i.e., containing 150 kb DNA) can escape from beads, this polymerizing activity must be attached. Nascent DNA, whether labelled *in vivo* or *in vitro* by short incubations with [^{3}H]thymidine or [^{32}P]TTP for 0.5 or 2.5 min, respectively, also resisted elution (13–15). These results are simply explained if polymerases are attached, directly or indirectly, to a large structure like a skeleton that cannot be removed electrophoretically.

4. Visualization of replication sites

4.1 Light microscopy

Seeing is believing; recently discrete sites of replication have been visualized. Rat fibroblasts in S phase were incubated with bromodeoxyuridine, then sites where the analogue had been incorporated were labelled using fluorescently tagged antibodies directed against the analogue. These sites were not diffusely spread throughout nuclei but concentrated in ~150 foci (16, 17, 36). Early during S phase the foci are small and discrete; later they became larger (18–21) when centromeric and other heterochromatic regions are being replicated (22). Permeabilized mammalian cells (Fig. 3) (23, 24) or demembranated frog sperm in egg extracts (25–28) incorporate biotin-labelled dUTP into analogous foci, visualized in this case with fluorescently labelled streptavidin or appropriate antibodies. These foci are not fixation artefacts because similar foci are seen after incorporation of fluorescein–dUTP into permeabilized, but unfixed, cells (29). The foci remain even when most chromatin is removed (17, 24).

Surprisingly, *Xenopus* extracts will organize pure DNA from phage lambda into foci and replicate their DNA (30) (see Chapter 6); this must mean that the extract contains all the necessary signals. Quite unexpectedly, glycogen, present in high concentrations in the extracts, plays some role in focus formation (31).

A single replication fork could not incorporate sufficient labelled analogue under these conditions to allow detection, so many forks must be active in each focus. As we know the number of foci, the rate of fork progression, the spacing between forks, the size of the genome, and the length of S phase, we can calculate that ~40 forks must be active in each of the small foci found early in S phase. If polymerases track, the 40 loops would have to be confined in some way to one small part of the nucleus; if fixed, the enzymes must be locally concentrated on an underlying structure. Indeed, particles containing the requisite number of polymerases can be extracted by lightly disrupting somatic nuclei (32), whilst more vigorous procedures give the simpler isolates usually studied by biochemists (33).

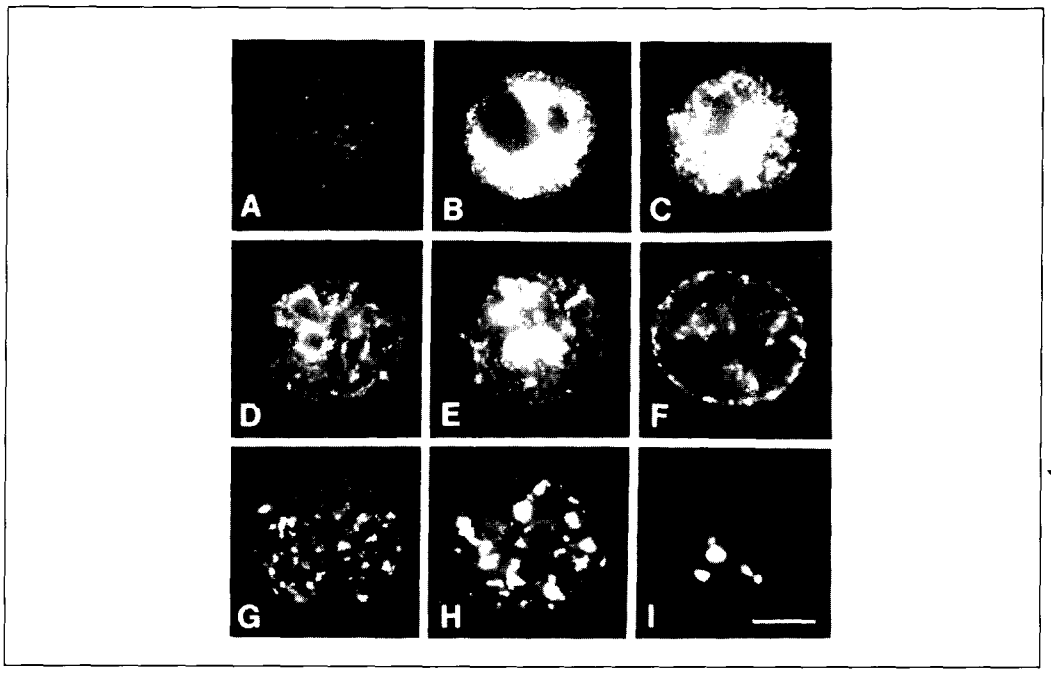


Fig. 3 Fluorescence micrographs of replication patterns found at different stages of S phase. Synchronized HeLa cells were encapsulated in agarose, permeabilized, incubated with biotin–dUTP and incorporation sites indirectly immunolabelled. Fluorescence marks replication sites, which change in number and distribution as cells progress from (A) early to (I) late S phase. Bar: 5 μm. From Hozák *et al.* (40) with permission of the Company of Biologists Ltd.

4.2 Electron microscopy

An early S-phase focus is ~200 nm in diameter, a size that is at the limit of resolution by light microscopy. Many attempts have been made over the years to localize replication sites by electron microscopy. For example, early autoradiographic studies showed that living cells incorporated [^{3}H]thymidine into dispersed chromatin, close to variably sized masses of condensed chromatin (34, 35). But as the pathlength of β-particles is many tens, even hundreds, of nanometers long, autoradiography does not provide sufficient resolution to allow precise localization. Moreover, nascent DNA could well move away from its synthetic site during the relatively long labelling times required to incorporate sufficient label to be detected. The same is true of experiments in which synthetic sites are immunolabelled after incubating living cells for 5 min or more with bromodeoxyuridine (22, 26).

4.2.1 Replication factories

If permeabilized cells are incubated with biotin–dUTP under suboptimal conditions which limit elongation to a few nucleotides, replication sites can be immunolabelled with gold particles to a higher resolution; the centres of gold particles are now con-

nected through an antibody bridge to the incorporated biotin and lie within 20 nm of it. In the first such experiments, most obscuring chromatin was also removed as described in Fig. 2 before 400 nm thick (resinless) sections were viewed in the electron microscope (Fig. 4). Residual clumps of chromatin could be seen attached to a 'diffuse skeleton' that ramified throughout the nucleus. (Although this network is morphologically complex, it contains lamin proteins, which are members of the intermediate-filament family; its 'core filaments' have the axial repeat typical of the family (37) and its nodes can be immunolabelled with anti-lamin antibodies (38). The lamins were named because it was originally thought that they were confined to the nuclear periphery; however, it now seems that the dense chromatin normally obscures the internal lamins.) Electron-dense bodies were scattered along this diffuse skeleton. They are present in the same numbers as the foci seen by light microcopy and during early S phase they are relatively constant in size (100–300 nm diameter). After elongating nascent DNA by ~500 nucleotides, gold particles were associated mainly with these electron-dense bodies (Fig. 4). As the incubation time was progressively increased, longer pieces of DNA were made and gold particles were found progressively further away from the dense bodies. This implies that nascent DNA is extruded from the dense body as templates pass through it.

As cells progress through S phase, these dense structures change in numbers, size, shape, and distribution just like the foci seen by light microscopy. Each contains ~40 active forks and associated leading- and lagging-strand polymerases, so it seems appropriate to call them replication 'factories'.

4.2.2 Factories are a subset of nuclear bodies

The factories seen in thick resinless sections correspond to a subset of the nuclear 'bodies' that have been seen over the years in conventional (thin) sections (34, 39); nuclear bodies are sites where biotin–dUTP is incorporated, they contain PCNA and they change in number, shape, and distribution just like factories (40).

4.3 Extra-factory replication

Although most replication takes place in factories, there is some extra-factory synthesis which increases as cells progress through S phase (40). There are special topological problems associated with replicating the last few basepairs between two replicons (41, 42) and this is true even if the two replicating forks are not immobilized (43). It is attractive to suppose that extra-factory labelling reflects a 'tidying-up' duplication of this hitherto unreplicated DNA.

5. Molecular content of replication factories

5.1 Attachment of origins

Ever since the replicon model was proposed, there has been discussion as to whether origins might be attached permanently to some underlying structure (44, 45).

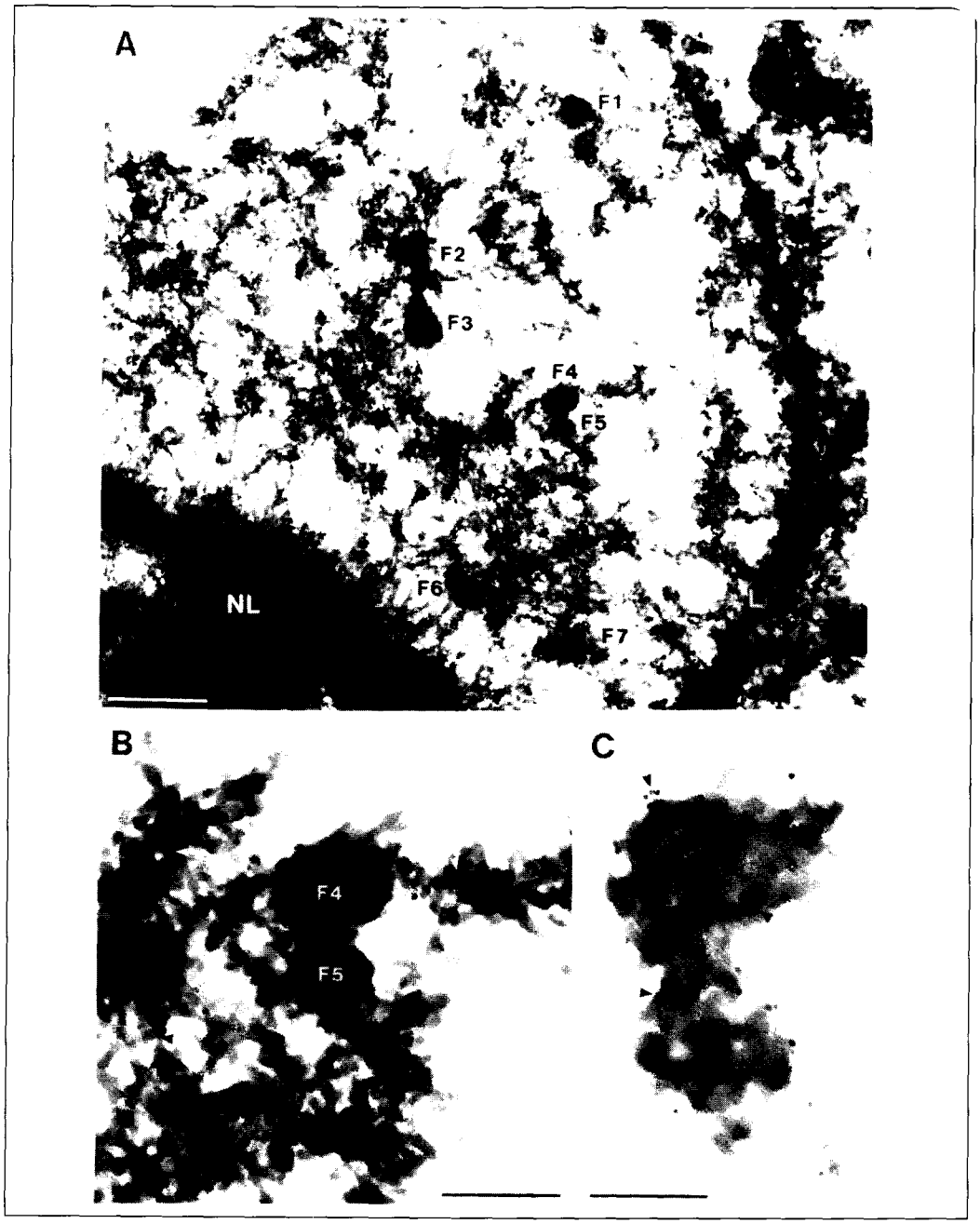


Fig. 4 Replication factories. Encapsulated cells were permeabilized with streptolysin, incubated with biotin–dUTP for 2.5 min, treated with nucleases, ~90% chromatin eluted, and sites of biotin incorporation immunolabelled with 5 nm gold particles. From Hozák *et al.* (24) with permission of Cell Press. (a) Seven replication factories (F1–7). NL: nucleolus. L: nuclear lamina. Of the 180 gold particles in the nuclear region (not visible at this magnification) 72% were in factories, indicating that factories were the site of DNA synthesis. Bar: 0.5 μm. (b) Higher-power view of F4 and F5. Three arrowheads point to the only extra-factory particles. Bar: 0.2 μm. (c) Underexposure and further 2 × magnification of F4 and F5 to show labelling; three arrowheads indicate some of the 30 gold particles. Bar: 0.1 μm.

However, experiments addressing this problem used unphysiological conditions which generate artefactual attachments of the chromatin fibre to the underlying skeleton (46); therefore, we still await a decisive answer to this question.

5.2 Proteins

Various proteins have been found in replication foci/factories including those specifically involved in synthesis as well as others that might be involved in regulation.

- DNA polymerase α (19, 24);
- PCNA (19, 23, 24);
- RP-A (47);
- cyclin A, cdk2, and RPA70, but not cyclin B1, cdc2, or RPA34 (48, 49). Cyclins are of interest because they may play a role in initiation (Chapters 6, 7, and 8).
- lamin B (50);
- DNA methyltransferase (51). This enzyme methylates DNA, so affecting gene expression. The presence of the enzyme at the replication site suggests that a methylation pattern, and so a pattern of gene expression, might be inherited by progeny duplexes. This transferase also contains a sequence that targets it to replication foci so similar sequences may target other proteins to replication sites.
- Various viral and cellular proteins have been found in the analogous 'factories' involved in viral replication; examples involving herpes virus include the virally encoded single-strand binding protein, ICP8 (52, 53), the cellular single-strand binding protein, as well as PCNA, Rb, p53, DNA ligase I, and DNA polymerase α, but not snRNP, c-Myc, p68, nucleophosmin (B23), or Ki67 (54).

6. Replication and transcription

Several tantalizing pieces of evidence suggest that RNA polymerases play a role in initiation (55).

Eukaryotic origins are always closely associated with transcription units and they are rich in binding sites for transcription factors which directly influence initiation (56, 57).

A temperature-sensitive mutant of BHK cells (i.e., tsAF8) arrests in G1 at the nonpermissive temperature, but microinjection of RNA polymerase II allows progression into S phase (58). A phenotypically similar mutant can also be rescued by transfection of a cDNA encoding the cell cycle gene 1 (*CCG1*) which turns out to encode a transcription factor associated with the TATA-binding protein, $TAF_{II}250$ (59–61). Another mutant can be rescued from G1 arrest by a human cell cycle gene, *BN51*; this is homologous with the yeast *RPC53* gene that encodes a subunit of RNA polymerase III (62). It can hardly be fortuitous that so many cell cycle mutants are deficient in polymerases and transcription factors unless transcription is critically involved in replication.

Discrete sites of transcription, which are analogous to replication sites, can be immunolabelled after incubation with Br-UTP (63, 64). There are ~300 such sites in a HeLa nucleus during G1 phase, each one containing ~40 active RNA polymerases. On entry into S phase, the ~300 sites aggregate to colocalize with the ~150 sites of replication and even late during S phase replication sites remain transcriptionally active (65, but see also 66). Again it seems unlikely that sites of replication and transcription would be so closely associated unless transcription plays some role during replication.

7. Repair replication

The repair of damage induced in DNA by ultraviolet light involves excision of the damage and then repair synthesis to fill the gap. Early work suggested that such repair synthesis both did, and did not, take place on an underlying skeleton (67–69). Sites of repair synthesis have now been immunolabelled after incorporation of biotin–dUTP; again they are not diffusely spread throughout nuclei but concentrated in discrete foci (70, 71). The repair activity seems not to be as closely associated with the nucleoskeleton as the S-phase activity; after treatment with an endonuclease as in Fig. 2, most repaired DNA and the repair foci are removed from beads with the chromatin fragments. However, as electroelution destroys repair activity (but not the S-phase activity), repaired DNA might be attached *in vivo* through a polymerase that was removed by the procedure. So this approach has not allowed us to determine decisively whether repair sites are associated with a skeleton *in vivo*.

8. Models for replication by immobile polymerases

We are all familiar with models involving tracking polymerases and at first sight those with fixed enzymes seem more complicated. However, they are not more so, it is only that we are used to seeing the enzymes move rather than the template. Figs 5–7 illustrate three views of chromosome duplication by fixed enzymes. (See 72–74 for some earlier models.)

8.1 Low-power view (Fig. 5)

During G1 phase, we imagine that the chromatin fibre is looped by attachment to transcription factories that are, in turn, attached to a nucleoskeleton; euchromatin is usually organized into smaller loops than heterochromatin. Loops are tied either through transcription factors at promoters/enhancers, or through active RNA polymerases to the factories (75, 76). Late during G1, some as yet undefined signal (mediated by a kinase?) triggers assembly of a replication factory around about two transcription factories (55, 65). Here the structures involved in transcription play a role in replication by nucleating assembly of replication factories.

Two extreme models for the evolution of factories can be envisaged. In one (Fig. 5,

left), small replication factories (1–6) might quickly assemble at the beginning of S phase around transcription factories, which would generally be on the same chromosome, and immediately become active. As one factory replicates many loops, not all origins need to fire simultaneously. By mid S phase, some small replication factories (e.g., 1) would have become redundant; they would be disassembled and their components incorporated into medium-sized factories that arise by growth and fusion of smaller factories (e.g., 2, 3). By late S phase, disassembly of most factories and growth/fusion of a few would generate large factories (i.e., 4–6) which replicate most heterochromatin; they will always contain nascent DNA as they grow from smaller active factories. Factories might apparently 'move' along the chromosome as now-redundant components at one (inactive) end become soluble and then become incorporated into the other (active) end.

In a second model, factories might be built *ab initio* at new sites on the chromatin fibre at different stages during S phase (Fig. 5, right). For example, the small factories A1, A2, and A3 might be built before the medium-sized factory B, which in turn is built before the large factory C, and some signal must trigger assembly at the appropriate time. If assembly of large factories takes time, we should see (but do not) partially built, but inactive, factories (not shown). Although the kinetics of appearance and activity of factories supports the (simple) first alternative involving factory growth and fusion, it is probably premature to exclude the second (40).

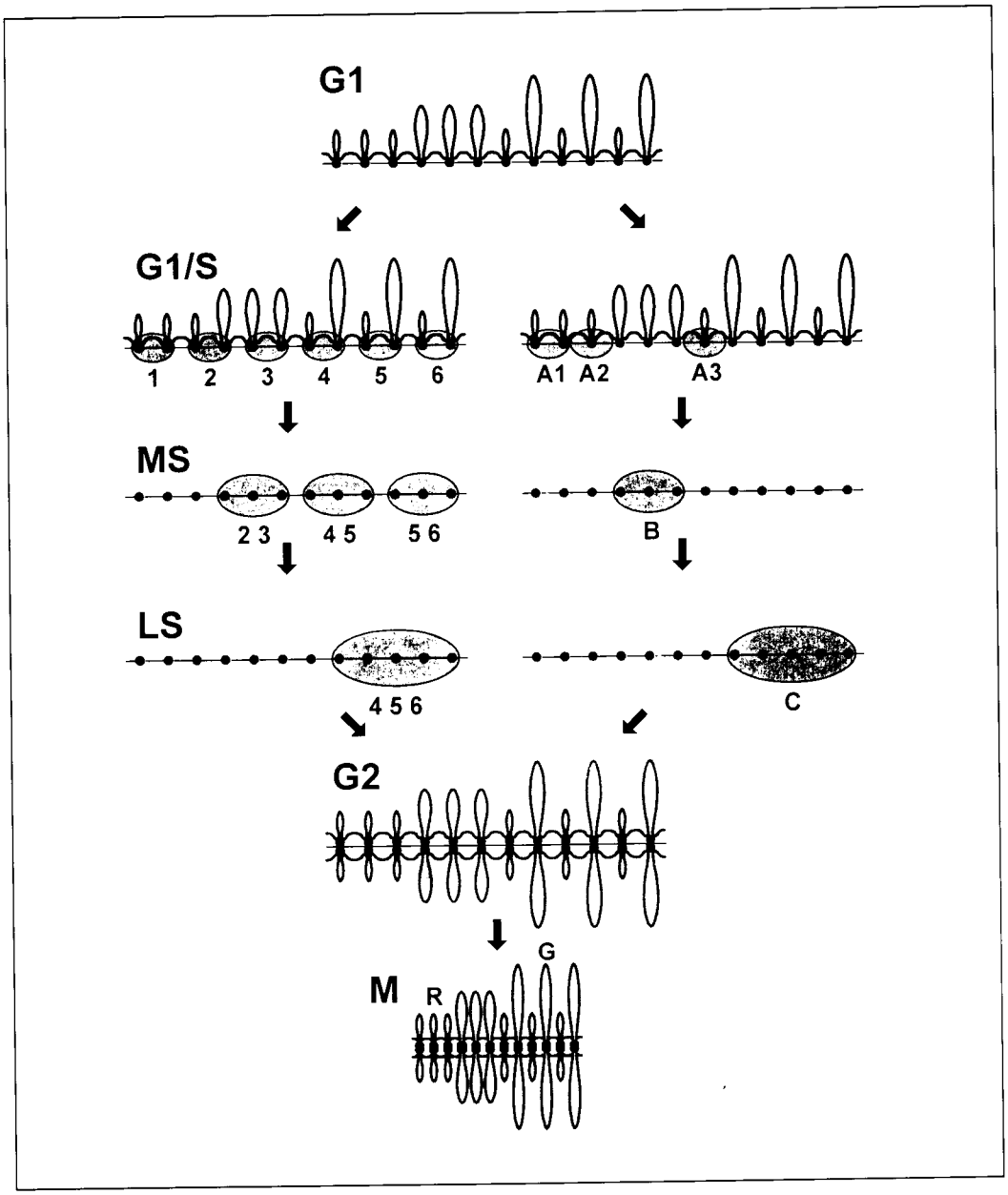
8.2 Medium-power view

Once a replication factory has been built, an origin within a loop will now lie close to polymerases on the surface of a factory and so will be likely to attach to one of them (Fig. 6; stage 1). Initiation occurs as strands within the origin separate (stage 2); then daughter duplexes are extruded (arrows), as parental duplexes slide (arrows) through the fixed polymerization site in the factory (stage 3). Replication continues (stage 4) as parental loops shrink (arrows) and daughter loops grow (arrows), until most DNA is duplicated (stage 5). Even the smallest factory replicates ~25 loops simultaneously. 'Tidying-up' replication of unreplicated DNA probably takes place later outside factories. Note that the origin is shown here to detach from the factory after initiation, but it may remain attached throughout the cycle (43).

8.3 High-power view

Figure 7 illustrates a model for the way that leading- and lagging-strand synthesis can be coordinated (see Chapter 1 for a model involving polymerases that are not fixed). DNA associated with a polymerizing complex (like the one illustrated in Fig. 6, stage 3), is shown in (a); it contains two replication forks. Parental duplexes slide into the symmetrical complex from each side (arrows). We will follow the path of the two grey segments behind the small open circles through the right-hand side of the complex. For clarity, the left-hand side has been omitted in panels (b)–(g), where only one fork is shown.

Continuous (leading-strand) synthesis is straightforward (illustrated at the bottom



of each panel); nascent DNA is extruded as the template slides past the fixed polymerase. (a) The 3′ end of the grey segment of the parental strand (marked by the small open circle) became single stranded as it slid past the helicase/topoisomerase; it has just reached the polymerizing site (bottom oblong). (b) The grey segment now slides past the polymerase (arrow) as nascent DNA is extruded into the bottom loop, enlarging it. (c) Copying of the grey segment has been completed.

Fig. 5 Models for chromosome duplication (low-power view). The G1 chromatin fibre is shown looped by attachment to transcription factories (circles) on a skeleton (horizontal line). Small and large loops represent euchromatin and heterochromatin respectively. For the sake of clarity, only one of the ~50 loops attached to each transcription factory during G1, G1/S, and G2, and none of those during mid and late S phase (MS and LS), are shown. Replication factories (ovals) are assembled around transcription factories and then most DNA is synthesized as templates slide through the factories, although some 'tidying-up' replication occurs outside factories to give the duplicated G2 fibre. On entry into mitosis (M), the skeleton disassembles (without changing the contour length of loops) and residual transcription factories (plus associated euchromatin and heterochromatin) collapse on to the chromosome axis to generate R and G bands. Two extreme models for the evolution of factories are illustrated. Left: Large factories grow from small factories. At the G1/S border, small replication factories (1–6) quickly assemble around pairs of transcription factories (generally on the same chromosome) which immediately become active. As one factory replicates many loops, not all origins need to fire simultaneously. By mid S phase, some small replication factories (e.g., 1) have become redundant; on disassembly their components are incorporated into medium-sized factories that arise by growth and fusion (e.g., 2, 3). By late S phase, disassembly of most factories and growth/fusion of a few generates large factories (i.e., 4–6) which replicate most heterochromatin; they are always labelled as they grow from smaller active factories. Factories might apparently 'move' along the chromosome as now-redundant components at one (inactive) end become soluble and then become incorporated into the other (active end). Right: Factories of different sizes are created *ab initio* at new sites at different times (first A1, A2, A3, then B, then C). If assembly of large factories takes time, we should see (but do not) partially built, but inactive, factories (not shown). Redrawn from Hozák *et al.* (40).

Lagging-strand synthesis is more complicated (illustrated at the top of each panel); because synthesis occurs 5′ → 3′ on strands of opposite polarity, the two strands must move locally past the relevant synthetic sites in opposite directions, which gives rise to a 'lagging-strand shuffle'. (a) The leading (5′) end of the grey parental strand (marked by the small open circle) became single stranded as it slid past the helicase/topoisomerase. (b) The 5′ end remains stationary as more of the grey segment slides into the complex (arrow), forming a loop. (c) A large loop has now formed. (d) The lagging (3′) end of the grey segment attached to the lagging-strand polymerase/primase (top oblong) and then slid 'backwards' (arrow) past it as an RNA primer was extruded. Both ends of the primer remain attached, the 3′ to the polymerase and the 5′ end to another site (arbitrarily shown here as part of the helicase/topoisomerase). DNA synthesis now begins as the 'backwards' flow continues (arrow). (e) A loop of nascent DNA is extruded as the segment continues to slide 'backwards' (arrow) past the polymerase (f) Copying of the grey segment to give the nascent Okazaki fragment is complete. (g) The loop containing the grey segment and its Okazaki fragment plus its primer rearranges, sliding past the 'processing' site (where gaps are sealed and primers removed) to enlarge the top loop. The cycle now repeats.

Models for initiation and termination involving fixed polymerases can also be drawn (43).

9. Conclusions

A wide body of results now suggests that replication occurs in eukaryotes as each template slides through a fixed polymerizing site and that many sites are organized into complex structures called 'factories'. It is easy to imagine how gently disrupting a cell might first release factories, before further disruption yields simpler polymerizing

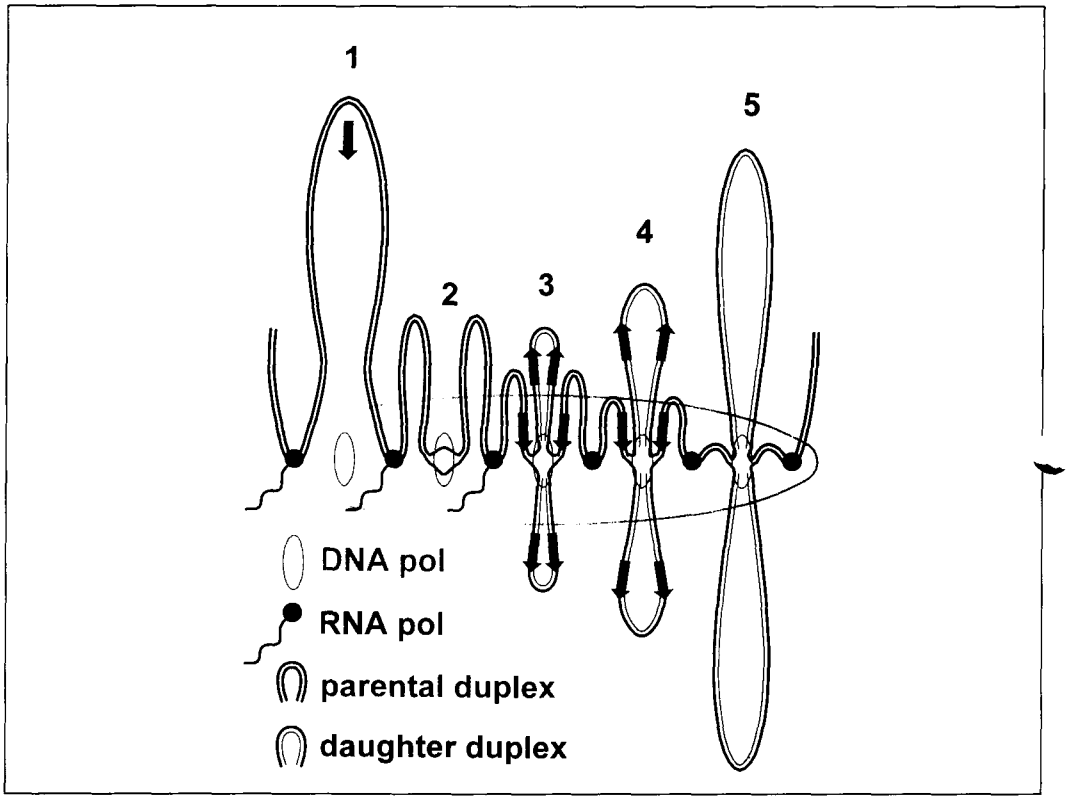


Fig. 6 A macroscopic model for replication (medium-power view). Various stages (1–5) in the replication of a chromatin loop, which is attached to a replication factory (large oval) through RNA polymerase/transcription factor complexes (filled circles); see text for details.

activities. Such pure enzymes might well track along templates *in vitro* but this does not necessarily mean that they do so *in vivo*. Immobilization in simple 'organisms' might be achieved by membrane attachment (e.g., in bacteria and mitochondria) or dimerization (e.g., in a virus), with a complex at one fork attached to, and immobilized by, its sister at the other. And if one kind of polymerase is immobile, it may be that other polymerases like those involved in RNA synthesis (77), reverse transcription (78), telomere synthesis, and recombination are also immobilized.

Immobilization of replicative enzymes in factories immediately suggests new and different mechanisms of control including:

(1) control of factory construction and activation;

(2) control of attachment of origins to the factory;

(3) restriction of subsequent attachments of the template to the factory to ensure that the template is replicated once, and only once, during the cell cycle; then structures, rather than soluble components, might 'license' only one cycle of replication (26).

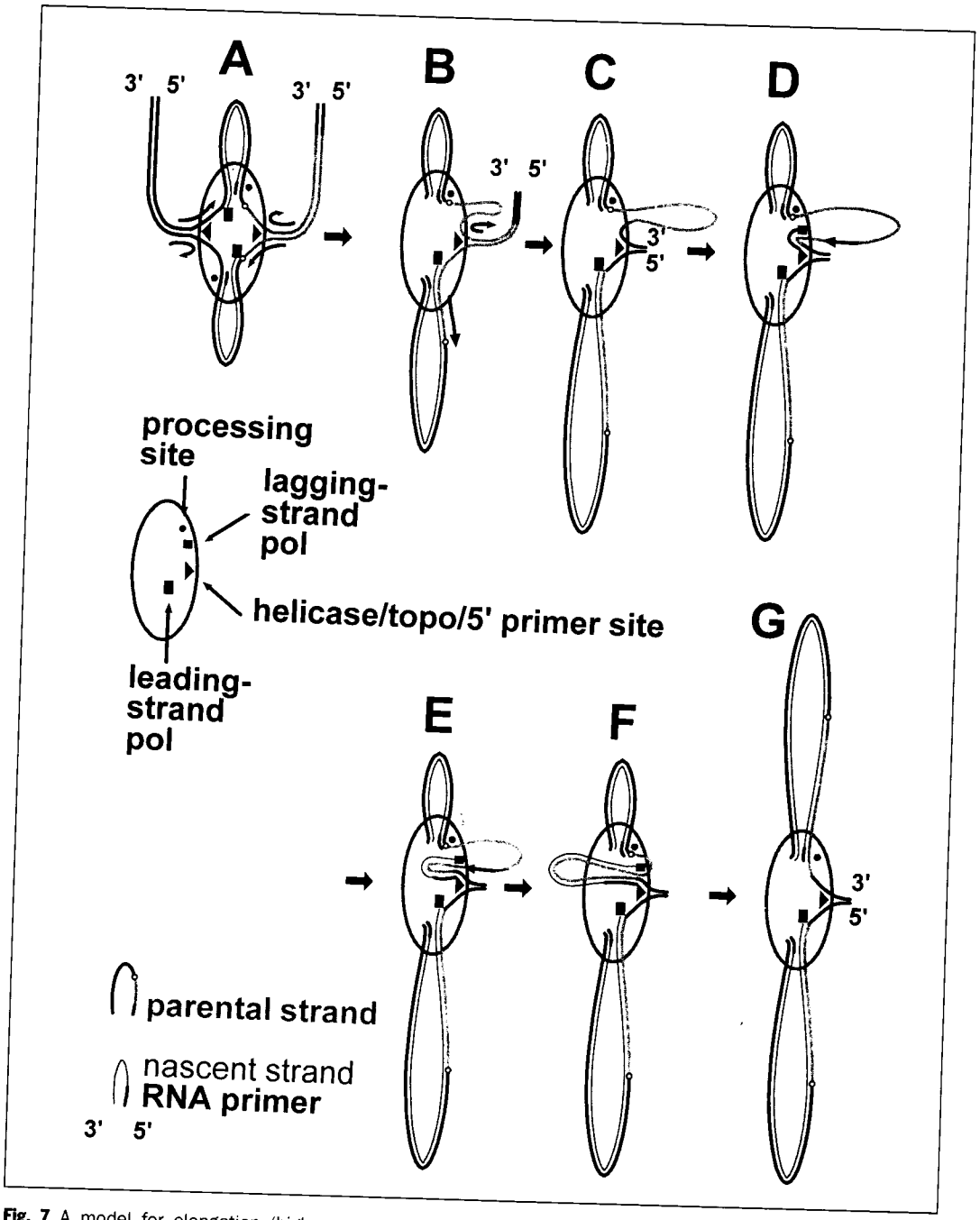


Fig. 7 A model for elongation (high-power view). Various stages during elongation are shown (see text for details). Redrawn from Cook (43).

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