Replication factories

Pavel Hozák and Peter R. Cook

During S phase, DNA replication begins at numerous sites throughout the genome. Textbooks would have us believe that each replication fork tracks along the immobile DNA until it runs into the adjacent fork, but recent results question this view. Various studies show that replication forks are concentrated in immobile 'factory' units throughout the nucleus. Each factory contains as many as 40 different replication forks and associated polymerases. These findings suggest that newly synthesized DNA is extruded as each template moves like a conveyor through the factory.

The idea that DNA polymerases track along the template DNA pervades our thinking[1] (Fig. 1a–c). It stems from a perception of relative size: the smallest object - the polymerase – moves. As is the case for many received ideas, there is little direct evidence for this view. In fact, recent evidence suggests the template moves past a fixed polymerase, rather than vice versa (Fig. 1d–f). Discussions here will concentrate on eukaryotic enzymes, but as the catalytic domains of all polymerases are probably structurally related[2], it seems likely that they all work similarly.

Jacob *et al.*[3] first suggested a role for the bacterial membrane in regulating DNA synthesis and distributing DNA to daughter cells; they proposed that attachment of a chromosome to the membrane might regulate initiation and that specific growth of the membrane between two attached progeny chromosomes would ensure the correct segregation of the chromosomes to daughter cells. While membranes do seem to play several distinct roles in these processes, precise mechanisms remain obscure[4,5].

The suggestion of Jacob *et al.* led to a search for analogous membrane attachments in eukaryotes. This search was eventually widened to include attachments of nascent DNA to nuclear 'matrices' and 'cages' and resulted in an extensive, but controversial, literature[6]. The evidence for such attachments is subject to the criticism that the associations seen are artifactually induced by unphysiological procedures used to isolate the nuclear substructures[7]; nascent DNA is very sticky and polymerases are known to aggregate during extraction in the high salt concentrations used[8]. This criticism can be countered only by analysing structures *in vivo* or, failing that, using conditions as close to the physiological as possible.

However, there was another important reason why the idea that polymerases are fixed was not widely accepted: replication could be reconstructed *in vitro* using pure soluble enzymes in the (apparent) absence of any immobilizing agents. For example, highly purified polymerases and auxiliary proteins replicate naked SV40 DNA authentically in the presence of the viral T antigen[9]. If soluble enzymes work satisfactorily, then there seems to be no need to suggest that they are immobilized by attachment to some hypothetical larger structure. And if they are smaller than the template, surely they must track along it.

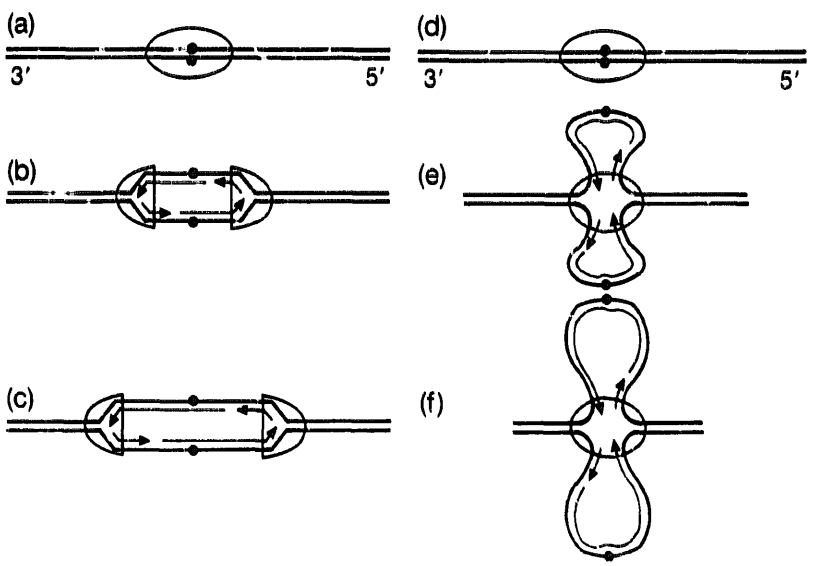

FIGURE 1

Models for replication involving mobile (a–c) and immobile (d–f) polymerases. The conventional model (a–c) involves binding of the polymerizing complex (grey oval) to the origin (black dots) (a), before the complex splits into two and the two halves track along the template (thick lines) as nascent DNA (thin lines) is made (b,c). Arrows indicate direction of growth of nascent chains. The alternative model (d–f) 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. When T antigen acts as a helicase, the structure in part f (without nascent DNA) is obtained, as well as the analogous structure in part c.

Fixed helicases

This argument for tracking by DNA polymerases has recently been compromised by the results of a simple, but important, experiment[10]. A helicase is required at each replication fork to convert double-stranded DNA to the single-stranded template on which the polymerase acts. Multimeric complexes of T antigen play this role during the replication of SV40. When T antigen was added to a linear template carrying the SV40 origin of replication, electron microscopy showed that a dodecameric complex of T antigen bound initially at the origin (as in Fig. 1a). In the presence of ATP (and a single-strand-

binding protein used to stabilize unwound single strands), the expectation was that the dodecamer would split into two, and then the two halves would track away from each other, unwinding the template to generate a 'bubble' with a hexamer at each fork (much as in Fig. 1b and c). However, although hexamers were associated with 75% of the unwinding intermediates, the remainder contained dodecamers at the base of two single-stranded loops (much as in Fig. 1e and f). Moreover, the formation of the unwinding intermediates could be stimulated at least fivefold by preincubating T antigen with a monoclonal antibody directed against the viral protein, and then 77% of the intermediates were associated with intact dodecamers. So in this simple system involving pure reagents, a significant number of forks are attached to, and so immobilized by, their partners. Any polymerase that acts at such an immobilized fork would be expected to be immobilized too. Therefore, the evidence from *in vitro* studies for a tracking polymerase is not as secure as it initially seemed to be.

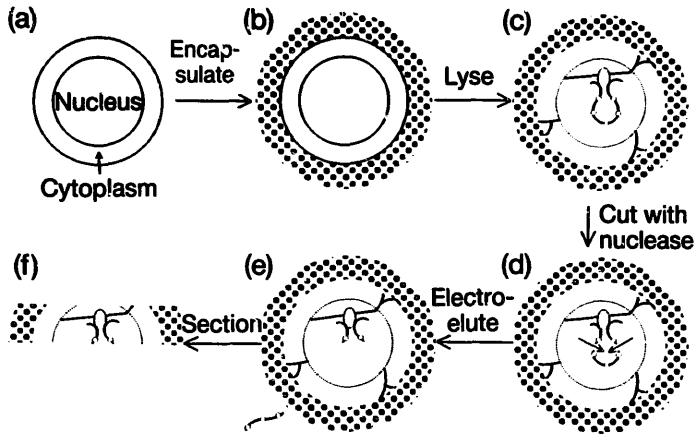

FIGURE 2

Procedure for visualizing nucleoskeletons and associated structures. (a–c) Cells are encapsulated in agarose (dotted surroundings) and lysed 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). (d–f) The dense chromatin obscures the nucleoskeleton but can be removed by cutting the chromatin fibre with a restriction enzyme, and then removing any unattached fragments electrophoretically. After cutting thick sections, the nucleoskeleton and associated factory can now be seen in the electron microscope.

Methodological advances

More-direct evidence for an immobile polymerase depended on three apparently unrelated methodological advances. The first allowed the use of conditions during biochemical analysis that are close to those present *in vivo*. Unphysiological conditions had been used previously to isolate structures like nuclear 'matrices' and 'cages' because chromatin aggregates into an unworkable mess at isotonic salt concentrations. More-physiological conditions can be used 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 continue to grow in standard tissue-culture media. After permeabilization with a mild detergent in a 'physiological' buffer, most cytoplasmic proteins and RNA diffuse out to leave an encapsulated cytoskeleton that surrounds the chromatin (Fig. 2a–c). These cell remnants are protected by the agarose coat but accessible to probes like antibodies and enzymes. While one cannot be certain that any isolate is free of artifacts, the fact that the integrity of the template and essentially all the replicative activity of the living cell can be retained in this system makes it unlikely that polymerases aggregate artifactually.

Models involving mobile or immobile (i.e. attached) polymerases can be distinguished by cutting the encapsulated chromatin with an endonuclease and then removing electrophoretically any unattached fragments, as illustrated in Fig. 2. If polymerizing complexes are attached to a large structure, they should remain in the agarose bead after electroelution; if unattached, they should electroelute from the bead with the eluting chromatin fragment. Chromatin containing DNA fragments of 150 kb can escape from beads, and in these experiments the chromatin was cut into pieces of <10 kb. Cutting chromatin in unsynchronized and permeabilized HeLa cells, followed by electroelution of 75% of the chromatin, hardly reduced DNA polymerizing activity; nascent DNA (whether labelled *in vivo* or *in vitro* in short pulses of 0.5 or 2.5 min, respectively) also resisted elution[11–13]. These results are simply explained if polymerases are attached, directly or indirectly, to a very large structure like a skeleton.

Seeing is believing. The second methodological advance allowed the visualization of sites of replication. Rat fibroblasts in S phase were incubated with bromodeoxyuridine and then sites of incorporation were visualized by the use of fluorescently labelled antibodies against the analogue. Approximately 150 foci were seen[14,15]. Early during S phase the foci were small and discrete; later they enlarged. Permeabilized mammalian cells or demembranated frog sperm in egg extracts incorporated biotin-labelled dUTP into analogous foci, visualized in this case with fluorescently labelled streptavidin or appropriate antibodies (see for example Refs 16 and 17). These foci are not fixation artifacts because similar foci are seen after incorporation of fluorescein–dUTP into permeabilized, but unfixed, cells[18]. The foci remain even when most chromatin is removed[15,17].

The visualization of focal sites of replication was a surprise; if polymerases tracked along the template we might have expected sites to be diffusely spread. A single replication fork could not incorporate sufficient labelled analogue under these conditions to allow detection, so many must be active in each focus. Indeed, we can calculate that about 40 forks must be active per focus from the number of foci, the known rate of fork progression, the spacing between forks, the size of the genome and the length of S phase. What underlying structure concentrates polymerases in a focus and organizes foci in a nucleus?

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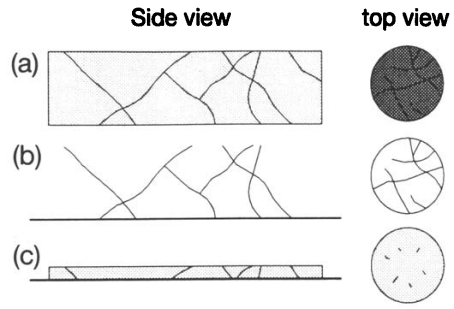
BOX 1 – SECTIONS USED FOR ELECTRON MICROSCOPY



Imaging depends on a difference in contrast between the biological sample and its surroundings. The conventional surroundings – the plastic resin used for embedding – provide little contrast, so samples are generally stained with heavy metals. Skeletal structures in cells are best viewed in thick sections (a; side view), but then it is difficult to resolve them against the background of resin (a; top view). Thinner sections of about a sixth of the thickness give less background, but only small regions of the skeleton can be seen (c). Thick 'resin-less' sections provide a solution[19]: samples are embedded in a wax to provide support during sectioning, before the wax is removed (b); now the skeletal elements in a thick section are not obscured.

An early S-phase focus is ~200 nm in diameter, a size that is at the limit of resolution by light microscopy. The third methodological advance allowed the ultrastructural visualization of replication sites. The resin sections generally used for electron microscopy are ill-adapted to analysing diffuse structures like skeletons, but Penman and colleagues[19] developed an important new method that did away with the resin (see Box 1). In this method, after fixation of the sample, embedding in a wax and sectioning, the wax is removed by organic solvents before viewing. Because there is no obscuring resin, much thicker sections (i.e. ~500 nm) can be used to visualize structures in three dimensions. In the nucleus, the dense chromatin generally obscures any underlying structures but it can be removed by cutting and electroelution. (High-resolution scanning techniques are also becoming increasingly useful for looking hundreds of nanometres into the nucleus from the periphery[20].)

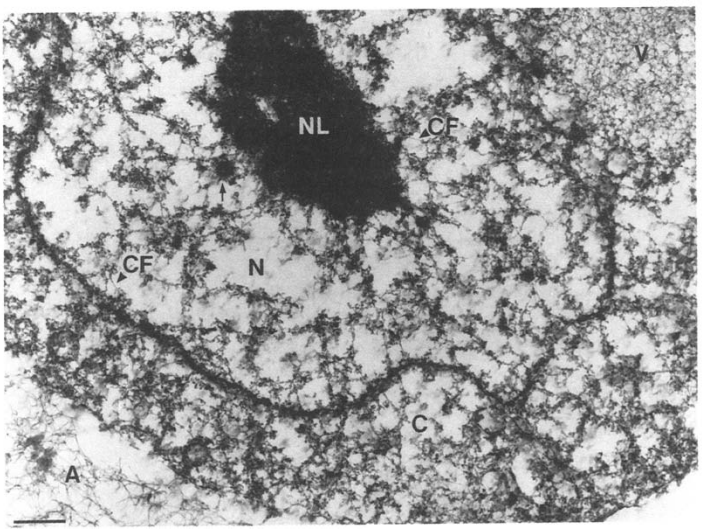


FIGURE 3

Electron micrograph of a HeLa cell in early S phase, from which ~90% chromatin has been removed. Encapsulated cells were permeabilized and treated with nucleases, then chromatin was eluted and a 400 nm resin-less section prepared. Residual clumps of chromatin and dense factories can be seen attached to a diffuse skeleton that ramifies throughout the nucleus. A, surrounding agarose; C, cytoplasmic region; V, vimentin-rich area; N, nuclear region; NL, nucleolus; CF, core filament; arrow, factory. Bar, 0.5 μm. Reproduced, with permission, from Ref. 17.

Replication factories

Sites of replication have now been visualized by the use of a combination of these techniques[17]. HeLa cells in early S phase were encapsulated in microbeads, permeabilized and incubated with biotin-labelled dUTP; then the chromatin was cut with nucleases and any detached fragments were electroeluted as in Fig. 2. Thick resin-less sections of such chromatin-depleted nuclei show residual clumps of chromatin attached to a 'diffuse skeleton'; in addition, 'core filaments', which have the same morphology as the intermediate filaments in the cytoplasm[21], ramify throughout the nucleus (Fig. 3). Electron-dense bodies are also scattered along the diffuse skeleton. They are present in the same numbers as the foci seen by light microscopy, and during early S phase they are relatively constant in size (100–200 nm diameter).

After incubation with biotin-labelled dUTP for 2.5 min, sites containing incorporated biotin were immunolabelled with gold particles: particles were associated mainly with these electron-dense bodies (Fig. 4). As the incubation time was gradually increased, the particles were found progressively farther away from the dense bodies. This implies that nascent DNA is extruded as templates pass through polymerases fixed within the dense body. As each body probably contains ~40 active replication forks, each with a leading- and lagging-strand polymerase, we called them replication 'factories'.

These observations raise many questions. When are factories assembled and destroyed? Do factories become active as soon as they are made? Are the large fluorescent foci seen later during S phase composed of clusters of factories like those of early S phase or are they made of larger structures?

Between 300 and 500 discrete and analogous sites of transcription have also been immunolabelled after incubating permeabilized HeLa cells with Br-UTP, by the use of an antibody that reacts with Br-RNA[22]. Each focus probably contains ~60 active RNA polymerases and many transcription units. Like the replication foci, they also remain when most chromatin is removed. Intriguingly, they change in size and distribution at the G1–S boundary to colocalize with replication sites[23]. This provokes more questions. Are there transcription 'factories' analogous to the replication factories? Are the machineries for replication

and transcription always found in separate factories, or are they found in one factory?

Immobile polymerases

Taken together, these results suggest that replication occurs *in vivo* as each template slides through a fixed polymerizing site and that many sites are organized into complex structures, which we call 'factories'. It is easy to imagine how an initial disruption of a cell would first release factories, before further disruption would yield simpler polymerizing activities. Indeed, particles containing the requisite number of polymerases can be extracted by lightly disrupting somatic nuclei[24], while more vigorous procedures give the simpler isolates usually studied by biochemists[5]. Such pure enzymes may well be able to track along the template but, as we have seen, 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.

It is possible to imagine how the processes of initiation, elongation and termination can all take place if the polymerase is immobile[25]. Immobilization immediately suggests new and different mechanisms of control. For example, we might expect the construction of factories during G1 phase to be very carefully regulated and that, once assembled, various factors might activate the factory. Once functioning, we can imagine how structural attachment of the template to the factory, rather than some soluble 'licensing factor'[26], could ensure that the template was replicated once, and only once, during the cell cycle. Moreover, if one kind of polymerase is immobile, are other polymerases like reverse transcriptases and RNA polymerases immobilized too[27,28]?

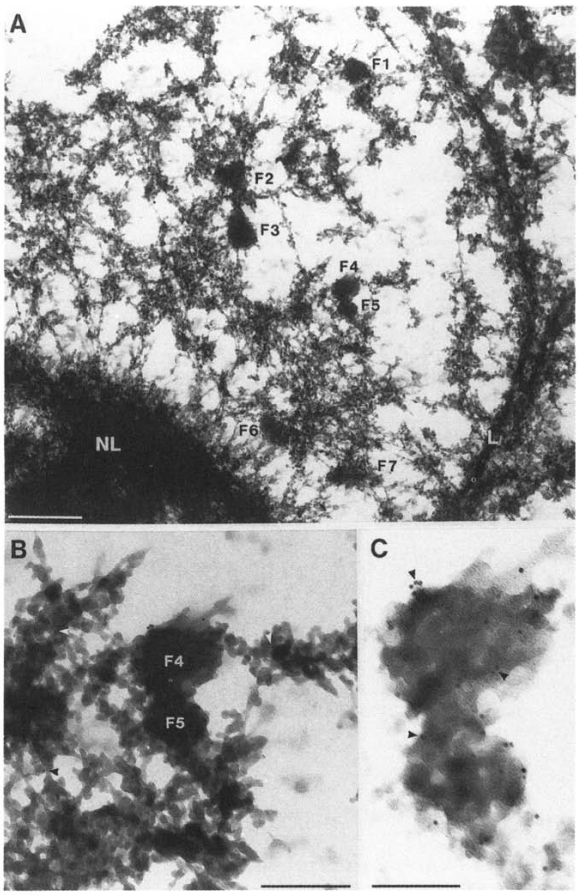


FIGURE 4

Factories are sites of replication. Encapsulated cells were permeabilized with streptolysin, incubated with biotin-11-dUTP for 2.5 min, and treated with nucleases; then ~90% of the chromatin was eluted and sites of biotin incorporation were immunolabelled with 5 nm gold particles. (A) Seven replication factories (F1–7). NL, nucleolus; L, nuclear lamina. 72% of the 180 gold particles in the nuclear region, which are not visible at this magnification, were in factories, indicating that they were the site of DNA synthesis. Bar, 0.5 μm. (B) Higher-power view of F4 and F5. Three arrows 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. Reproduced, with permission, from Ref. 17.

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Adenovirus entry into host cells: a role for α_v integrins

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The mechanism(s) by which nonenveloped viruses enter host cells is poorly understood. The recent identification of cell-surface α_v integrins as receptors for adenovirus internalization has shed much light on this process. In addition, analysis of α_v integrins as internalization receptors for adenovirus has provided further insights into the biology of integrins.

Since its isolation and identification as a major respiratory pathogen by Rowe *et al.*[1], adenovirus has been used widely to obtain fundamental knowledge in molecular and cell biology, including insights into RNA splicing[2], protein trafficking[3], and control of cell growth and immune functions[4,5]. The ability of adenovirus to infect a wide variety of cell types efficiently has also been exploited to deliver foreign genes into host cells[6]. Extensive information on the structure, protein composition and mode of replication of adenovirus has facilitated its use in determining the mechanisms by which nonenveloped viruses enter host cells.

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Molecular architecture of an adenovirus

A fascinating aspect of adenovirus structure is that the virion particle is designed not only for efficient packaging of the viral genome but also for efficient targeting and delivery of the viral genome into host cells. A detailed structural analysis of human adenovirus serotype 2 (Ad2) has been achieved by X-ray crystallography combined with image reconstruction of intact virus particles from cryoelectron micrographs[7,8]. Ad2 contains a 35 kb DNA genome that encodes at least 11 different structural proteins. Seven of these proteins assemble into a macromolecular complex that forms an icosohedral particle of 70–80 nm (excluding the fibre protein). Each of the 12 vertices of the virion contains a complex known as a penton, which comprises the penton-base and fibre coat proteins (Fig. 1). The fibre protein, a trimer of 62 kDa, is a highly extended molecule, 37 nm in length. Its N-terminus inserts into the penton base and the C-terminus mediates virus attachment to a host cell receptor[9]. The penton complex is circumscribed by five trimers of hexon, which are referred to as the peripentonal hexons. In addition to these coat proteins, adenovirus contains a number of minor proteins, which are probably mainly involved in stabilizing the capsid structure.

Pathway of adenovirus entry into host cells

High-affinity binding (K_d = 1–2 nM) of adenovirus to a host cell receptor is mediated by the fibre protein[10]. Although there is little information about the identity of the fibre receptor, preliminary characterization of the receptor on human epithelial cells indicates

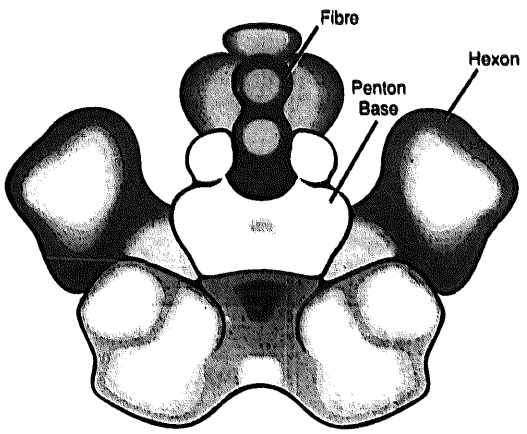

FIGURE 1

Schematic representation of the vertex region of human Ad2. The structure and location of the major Ad2 coat proteins – hexon trimers, penton base, and fibre – are depicted based on image reconstruction of cryoelectron micrographs in Ref. 7. Only the first third of the fibre is shown in this representation.