The Structural Basis of Nuclear Function

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Most models for transcription and replication involve polymerases that track along the template. We review here experiments that suggest an alternative in which polymerization occurs as the template slides past a polymerase fixed to a large structure in the eukaryotic nucleus—a "factory" attached to a nucleoskeleton. This means that higher-order structure dictates how and when DNA is replicated or transcribed.

KEY WORDS: Nucleoskeleton, DNA replication, Transcription, Repair, Chromatin loop.

I. Introduction

The cytoplasm is a complicated cellular compartment, with different regions dedicated to different functions. Various organelles and subcompartments are organized around different skeletons, and complex mechanisms direct molecular traffic between these subcompartments and surrounding membranes. In contrast with this complexity, the nucleus has traditionally been viewed as relatively unstructured, with few major compartments (e.g., the envelope, heterochromatin, euchromatin, and nucleoli). It is now being realized that the structure of the nucleus is as complex as that of the cytoplasm, with different skeletons and subcompartments, each with its own particular function. For example, the major nuclear processes of replication, the repair of damage in DNA, transcription, RNA processing, and transport, all take place in specific subcompartments. Moreover, complex controls manage traffic between these subcompartments and the cytoplasm. Therefore, nuclear architecture profoundly affects gene function. We review here its roles in replication, transcription, and the repair of damage

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in DNA. Work using conditions close to the physiological and that maintain as much of the functional integrity as is conveniently possible will be emphasized. The nuclear matrix and scaffold as described in some other chapters in these volumes are commonly isolated under less physiological conditions and hence may display more perturbed functional properties.

The appreciation of the role played by large structures in nuclei has led to a reevaluation of the mechanism of polymerization. The traditional view involved polymerases that tracked along the template (Figs. 1A–C and 2A). (The term polymerase is used here to describe the cluster of many different polypeptides that form an active complex in which the polymerizing subunits are present only as minor components.) This view was sensible if polymerases were small relative to the template and if they acted alone. However, recent experiments show that many DNA polymerases are concentrated together into large structures, "factories", that dwarf the template. Therefore we imagine that several templates slide past a number of DNA polymerases fixed within factories; the template moves rather than the enzyme (Figs. 1D–F). Evidence is also accumulating that transcription occurs similarly (Fig. 2B). Indeed, logic suggests that if the RNA polymerase did track along the helical template, the resulting transcript would be entangled about the template (Fig. 2B,upper); however, no satisfactory mechanism has yet been suggested as to how it might be untangled prior to export to the cytoplasm. A sensible alternative is to immobilize the polymerase;

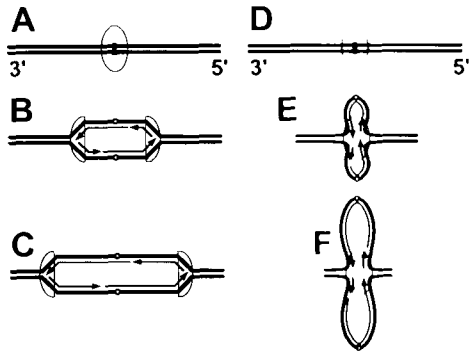

FIG. 1 Models for replication involving (A–C) mobile and (D–F) immobile polymerases. A–C. The conventional model involves (A) binding of the polymerizing complex (oval) to the origin (black dots), before the complex splits into two and (B,C) the two halves track along the template (thick lines) as nascent DNA (thin gray 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. [From Hozák *et al.* (1995a) with permission of Oxford University Press.]

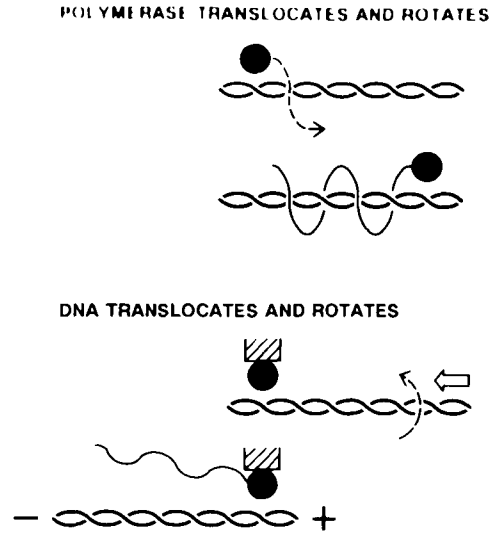

FIG. 2 Models for transcription involving (top) mobile and (bottom) immobile polymerases. Top. The polymerase (solid circle) tracks (arrow) along one of the two helical strands to generate a transcript. This transcript is entangled about the template and must be untangled, but no satisfactory mechanism has yet been suggested with regard to how this might be achieved [discussed by Cook and Gove (1992) and Cook (1994)]. Bottom. DNA rotates (arrow) as it slides (large arrow) past an immobile polymerase (solid circle attached to hatched area); the transcript is extruded as template rotation generates positive and negative supercoils (indicated by + and –) which must be removed by a topoisomerase. The movements involved are similar to driving a screw (the DNA) through a fixed nut (the polymerase). [From Cook (1994) with permission of ICSU Press.]

then an unentangled transcript can be extruded as the template moves both rotationally and laterally through the fixed polymerizing site (Fig. 2B, lower). This means that the position of a sequence in three-dimensional space relative to the fixed polymerases will dictate how easily it can engage a polymerizing complex (i.e., attach) so that replication or transcription can begin. As a result, higher order structure controls both processes. Although discussion here concentrates on eukaryotic enzymes, it seems that prokaryotic enzymes will work in much the same way, as the catalytic domains of DNA polymerases are structurally related to the eukaryotic enzymes (Braithwaite and Ito, 1993).

A. Artifacts

Isotonic salt concentrations are not generally used during nuclear fractionation or during polymerase assay because they cause chromatin to ag-

precipitate into an unworkable mess and, quite naturally, more tractable conditions are used instead. However, chromatin is poised in a metastable state and quite small changes in tonicity dramatically affect structure. For example, one tenth the physiological salt concentration—usually used to isolate nuclei and chromatin—destroys the 30-nm fiber, extracts a quarter of nuclear protein including DNA polymerases and generates a new attachment of the fiber to the substructure for every one that preexists. Often residual aggregation is suppressed by adding "stabilizing" cations like magnesium ions, spermine, or spermidine, but these generate further artifactual attachments. Then it is not surprising that slight differences in isolation conditions generate very different structures, each with its own characteristic set of sequences associated with a different subset of proteins. For example, matrix attached regions (MARs) are bound to different proteins depending on the precise method of isolation, scaffold attached regions (SARs) are often specifically associated with topoisomerase II, and transcribed sequences are bound to "cages." Skeptics point to the fact that even those in the field cannot agree on which sequences are associated with which proteins in a particular substructure and naturally suggest that some, or all, are isolation artifacts with no counterparts *in vivo* (Cook, 1988; Jack and Eggert, 1992).

Against this background, it is not surprising that convincing evidence for a role of larger structures in nuclear functions was only obtained with the use of more physiological conditions that preserved many nuclear functions.

B. Physiological Conditions

The practical problems caused by chromatin aggregation under physiological conditions can be overcome if cells are encapsulated in agarose microbeads (50–150-μm diameter) before lysis. Agarose is permeable to small molecules so encapsulated cells continue to grow in standard tissue-culture media. When they are permeabilized with a mild detergent in a "physiological" buffer most soluble cytoplasmic proteins and RNA diffuse out to leave the cytoskeleton and associated material, as well as the nucleus (Fig. 3A–C; Jackson and Cook, 1985a; Jackson *et al.*, 1988). The agarose protects these cell remnants and, importantly, the encapsulated nuclei can be manipulated freely without aggregation while they remain accessible to probes like antibodies and enzymes. As the template remains intact and as essentially all the replicative and transcriptional activity of the living cell is retained, it seems unlikely that polymerases could have aggregated after lysing the cell. Note that almost all attachments to be discussed below involve active polymerases.

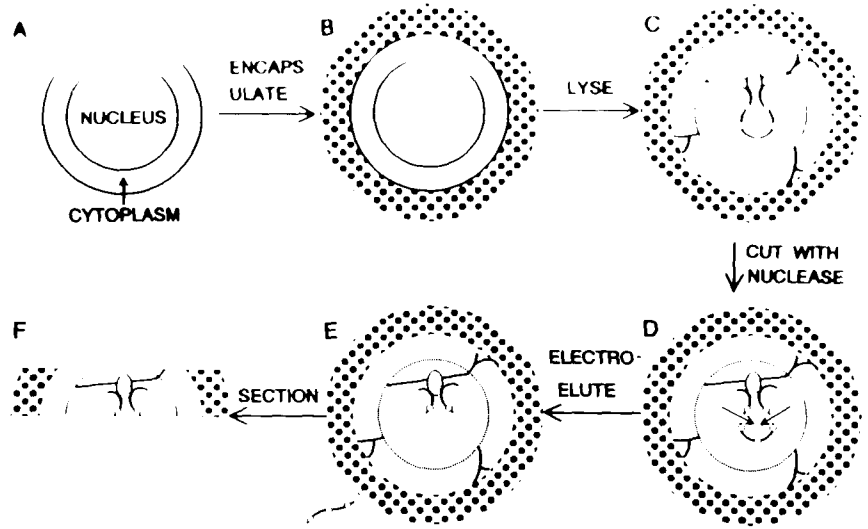

FIG. 3 Procedure for visualizing nucleoskeletons and associated structures. (A) Cells are (B) encapsulated in agarose (dots) and (C) lysed to leave a cytoskeleton, nuclear lamina (dotted circle), and nucleoskeleton (straight line) to which is attached a replication factory (gray oval) and a DNA loop covered with nucleosomes (open circles). Dense chromatin obscures the nucleoskeleton but can be removed by (D) cutting the chromatin fiber with a restriction endonuclease, 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 (1994) with permission of ICSU Press.]

II. Nucleoskeletons and Chromatin Loops

Most cell biologists now agree that chromosomal DNA is looped into domains by attachment of the chromatin fiber to a nucleoskeleton. Fortunately, meiotic lampbrush chromosomes of living newt cells provide undisputable proof of chromatin loops attached to a skeleton core (Callan, 1977). Unfortunately, ever since the initial demonstration of individual superhelical domains in "nucleoids" from interphase cells (Cook and Brazell, 1975), there has been little agreement as to what the molecular basis of the skeleton is and what the sequences involved in the attachments might be. As outlined above, controversy has centered on whether the attachments seen *in vitro* are also found *in vivo*.

It is important to recall that the existence of the cytoskeleton was once controversial largely because it proved so difficult to see; the widths of its individual elements are well below the resolution of the light microscope (i.e., ~100 nm) and the sections used for electron microscopy are usually

too thin to contain extended regions of the skeleton. That controversy evaporated once (1) individual elements of the skeleton were seen by fluorescence microscopy after immunolabeling and (2) complete skeletons were imaged by electron microscopy of whole mounts. So perhaps it is not surprising that the nucleoskeleton has proven equally difficult to image, especially since it is obscured by dense chromatin, which also prevents access of antibodies to it.

We have visualized a nucleoskeleton using the approach illustrated in Fig. 3. Encapsulated cells were lysed, the chromatin fiber cut with restriction endonucleases, and then most obscuring chromatin removed by electrophoresis. Electron microscopy of thick (resinless) sections (Capco *et al.*, 1982; He *et al.*, 1990) then revealed residual clumps of chromatin attached to a "diffuse skeleton" that ramified throughout the nucleus (Fig. 4). This network is morphologically (and functionally) very complex, but its "core filaments" had the axial repeat typical of the intermediate-filament family

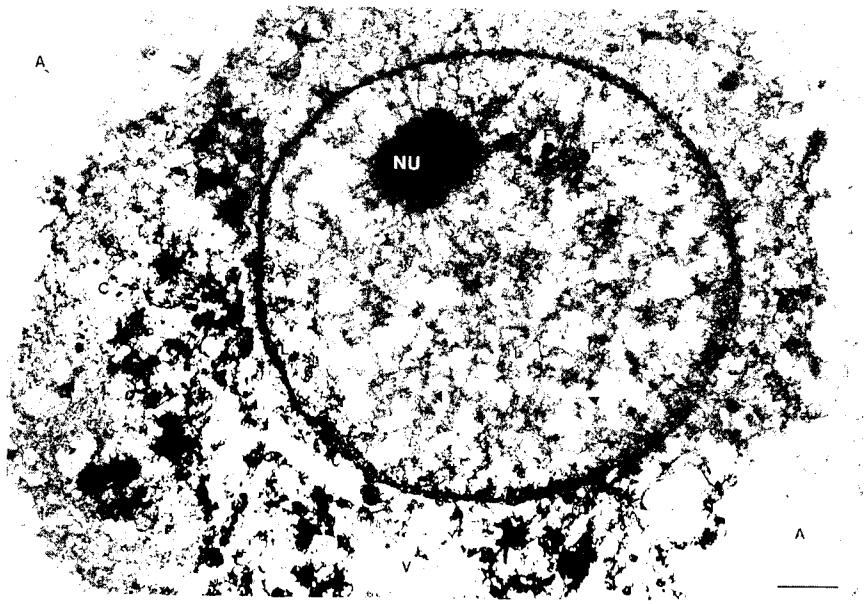

FIG. 4 Electron micrograph of cell 10 hr postmitosis from which ~90% chromatin has been removed. Encapsulated cells were permeabilized, incubated with biotin dUTP, treated with nucleases, chromatin eluted, incorporated biotin immunolabeled with 5-nm gold particles, and a 500-nm resinless section prepared. Agarose (A) surrounds cytoplasmic (C) and nuclear remnants where residual clumps of chromatin are attached to a diffuse network that ramifies from nucleolus (NU) to lamina. Gold particles, which are not visible at this magnification, were concentrated in replication factories (F). Bar: 1 μm. [From Hozák *et al.* (1994b) with permission of the Company of Biologists Ltd.]

[illegible] (Jackson and Cook, 1988). Immunofluorescence—using various antibodies, including one that specifically recognized an epitope in lamin A between amino acids 598 and 611, and cell types (i.e., HeLa, HEp 2, and SW13)—revealed internal "speckles" when the obscuring chromatin was removed (Hozák *et al.*, 1995b). Electron microscopy showed that all three antibodies labeled nodes on the diffuse nucleoskeleton, although the core filaments were weakly labeled (Hozák *et al.*, 1995b). One anti-lamin B2 antibody reacted weakly with foci on this diffuse skeleton in a minority of cells. Moreover, electron microscopy using (conventional) thin sections from which no chromatin was removed revealed as much lamin A in the interior as at the periphery.

This result was surprising. It is widely assumed that the nuclear lamins—as their name indicates—are confined to the nuclear periphery. However, they are occasionally found internally, for example during G1 phase, in certain pathological states, when mutated, or when overexpressed (e.g., Cardenas *et al.*, 1990; Gill *et al.*, 1990; Bader *et al.*, 1991; Beven *et al.*, 1991; Kitten and Nigg, 1991; Eckelt *et al.*, 1992; Goldman *et al.*, 1992; Lutz *et al.*, 1992; Mirzavan *et al.*, 1992; Bridger *et al.*, 1993). This internal location has been attributed to an accidental mislocation of proteins destined for the periphery, but could equally arise from an overconcentration at a normal site. The results described above suggest that the lamins have been misnamed; they have both a peripheral and an internal location.

We have also measured loop size using physiological conditions. Most chromatin was removed as before by treatment with an endonuclease followed by electrophoresis to leave residual clumps of chromatin attached to the nucleoskeleton (Fig. 3). An average loop size of 86 kb (in HeLa cells) was then deduced from the percentage of chromatin remaining and the size of the attached fragments (Jackson *et al.*, 1990). No large changes in loop size could be detected at different stages in the cell cycle. Therefore the fundamental attachments probably persist during the gross structural changes occurring during mitosis. Loop size measured in this way is, of course, an average; for a discussion, see Jackson *et al.*, (1990).

III. Attached Polymerases

Models involving tracking DNA and RNA polymerases can be distinguished from those involving immobile (i.e., attached) enzymes by cutting encapsulated chromatin into fragments of ~10 kb with an endonuclease and then removing electrophoretically any unattached fragments (Fig. 3; Jackson and Cook, 1985a,b; 1986a,b; Jackson *et al.*, 1988). If polymerases are attached to either directly to the skeleton or indirectly through a larger

structure which is in turn attached to the skeleton), they should remain in the agarose bead after electroelution; if unattached, they should electroelute from the bead with the eluting chromatin. Cutting chromatin in unsynchronized and permeabilized HeLa cells, followed by electroelution of >75% of the chromatin, hardly reduced DNA or RNA 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 and RNA—whether labeled *in vivo* or *in vitro* by short incubations with [^{32}P]TTP or [^{32}P]UTP—also resisted elution. These results are simply explained if polymerases are attached, directly or indirectly, to a very large structure like a skeleton.

A. Replication Factories

Seeing is believing; the direct visualization that replication takes place at a limited number of discrete sites in the nucleus has perhaps provided the most persuasive evidence of a role for an underlying structure. In the original experiment, rat fibroblasts in S-phase were incubated with bromodeoxyuridine; then sites where the analogue had been incorporated were labeled using fluorescently tagged antibodies directed against the analogue. These sites were not diffusely spread throughout nuclei but concentrated in ~150 foci (Nakamura *et al.*, 1986). Subsequently it was found that early during S-phase the foci were small and discrete; later they became larger (Nakayasu and Berezney, 1989; Fox *et al.*, 1991; Kill *et al.*, 1991; Humbert and Usson, 1992; Manders *et al.*, 1992) when centromeric and other heterochromatic regions were replicated (O'Keefe *et al.*, 1992). Permeabilized mammalian cells (Fig. 5; Bravo and Macdonald-Bravo, 1987; Hozák *et al.*, 1993) or demembranated frog sperm in egg extracts (e.g., Blow and Laskey, 1986, 1988; Hutchinson *et al.*, 1987, 1988; Mills *et al.*, 1989) incorporate biotin-labeled dUTP into analogous foci, visualized in this case with fluorescently labeled streptavidin or the appropriate antibodies. These foci are not fixation artifacts because similar foci are seen after incorporation of fluorescein-dUTP into permeabilized, but unfixed, cells (Hassan and Cook, 1993). The foci remain even when most chromatin is removed (Nakayasu and Berezney, 1989; Hozák *et al.*, 1993), implying that they are attached to an underlying structure.

Under the conditions used in these experiments, a single replication fork could not possibly incorporate sufficient labeled analogue to be detected, so many forks must be active in each focus; indeed, simple calculations (based on the number of foci, the rate of fork progression, the spacing between forks, the size of the genome, and the length of S-phase) show that ~40 forks must be active in each early S-phase focus in a human cell.

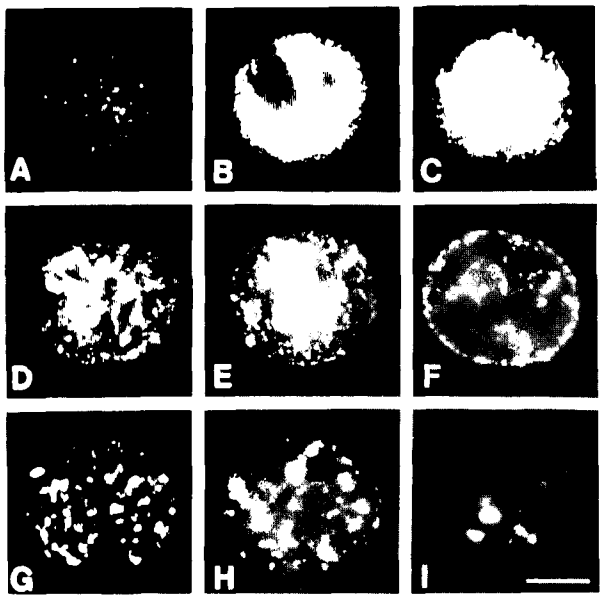

FIG. 5. Fluorescence micrographs of replication patterns found at different stages of S-phase. Synchronized HeLa cells were encapsulated in agarose, permeabilized, incubated with biotin-dUTP, and incorporation sites indirectly immunolabeled. Fluorescence marks replication sites, which change in number and distribution as cells progress from (A) early to (I) late S-phase. Bar, 5 μm. [From Hozák *et al.* (1994b) with permission of the Company of Biologists Ltd.]

This is simply explained if the 40 forks are attached to an underlying structure. Even if polymerases do track, some mechanism must confine the 40 tracking enzymes to one small part of the nucleus and, presumably, this would involve attachment of 40 templates to an underlying structure. So whatever the model, we must invoke some role for a substructure. However, as early S-phase foci have diameters of 100–200 nm, a size that is at the limit of resolution by light microscopy, it would be difficult to use fluorescence microscopy to characterize their relationship to such a substructure.

Many attempts have been made to localize replication sites by electron microscopy. Early autoradiography showed that living cells incorporated [^{3}H]thymidine over periods of many minutes into dispersed chromatin, close to variably sized masses of condensed chromatin (Bouteille *et al.*, 1974; Fakan, 1978). But as the path length of the β-particles emitted by ^{3}H is many tens, even hundreds, of nanometers long, this approach could not provide sufficient resolution for precise localization. Moreover, nascent DNA was made at a rate of about [illegible] nucleotides per second so that most of the incorporated label would have had plenty of time to move away from

its synthetic site. The same is true of experiments involving incubations of 5 min or more with bromodeoxyuridine rather than [^{3}H]thymidine (e.g., Mazzotti *et al.*, 1990; O'Keefe *et al.*, 1992).

Synthetic sites can be immunolabeled with gold particles to a much higher resolution after incubating permeabilized cells with a suboptimal concentration of biotin-dUTP to ensure that the incorporated analogue remains close to the polymerization site. Gold particles then lie within 20 nm of the incorporated biotin (i.e., connected through an antibody bridge). In the first such experiments, most obscuring chromatin was removed as described in Fig. 3 before 400-nm-thick (resinless) sections were viewed in the electron microscope. Electron-dense bodies were scattered along the diffuse nucleoskeleton; they were present in the same numbers as the foci seen by light microscopy, and during early S-phase they were 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. 6). 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 structures seen by electron microscopy change in numbers, size, shape, and distribution just like foci seen by light microscopy (Fig. 5). Replication foci/factories contain proteins specifically involved in synthesis (e.g., DNA polymerase α, PCNA, and DNA methyltransferase (Leonhardt *et al.*, 1992; Hozák *et al.*, 1993) as well as others that might be involved in regulation [e.g., cyclin A, cdk2, and RPA70, but not cyclin B1 or cdc2 (Adachi and Laemmli, 1992; Cardoso *et al.*, 1993; Sobczak-Thepot *et al.*, 1993)]. As even the smallest contain ~40 active forks and associated leading- and lagging-strand polymerases, it seemed appropriate to call them replication factories. Fragments of such factories were probably isolated by Tubo and Berezney (1987) when they isolated particles containing many polymerases by lightly disrupting somatic

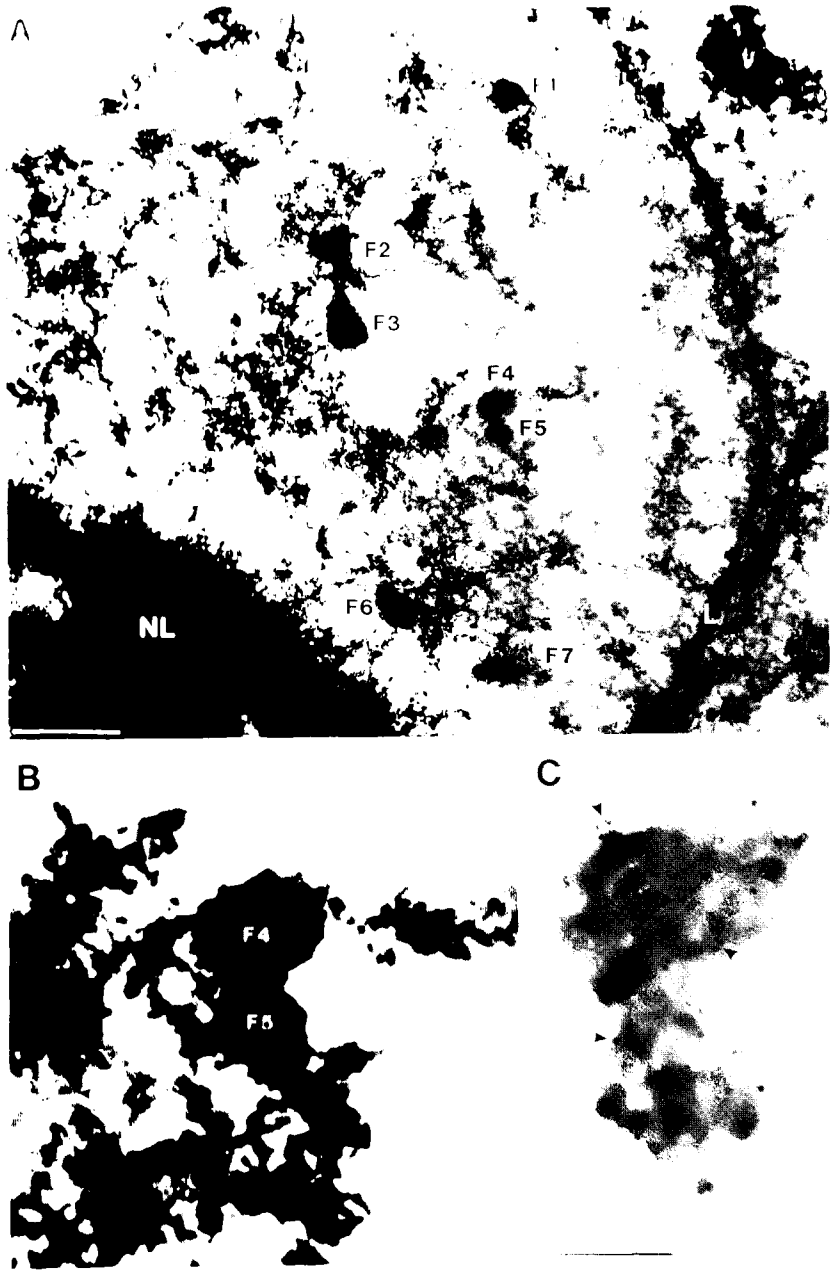

FIG. 6 Replication factories. Encapsulated cells were permeabilized with streptolysin, incubated with biotin-dUTP for 2.5 min, treated with nucleases, 90% chromatin eluted, and sites of biotin incorporation immunolabeled with 5-nm gold particles. [From Hozák *et al.* (1993) with permission of Cell Press.] A. Seven replication factories (F1–7). NL: nucleolus. L: nuclear lamina. Of the 180 gold particles in the nuclear region 72% (not visible at this magnification) were in factories, indicating that factories were the site of DNA synthesis. Bar: 0.5 μm. B. Higher-power view of F4 and F5. Arrowheads point to the only extrafactory particles. Bar: 0.2 μm. C. Underexposure and further 2× magnification of F4 and F5 to show labeling; arrowheads indicate some of the 30 gold particles. Bar: 0.1 μm.

nuclei, while more vigorous procedures give the simpler isolates usually studied by biochemists.

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 (Bouteille *et al.*, 1974; Brasch and Ochs, 1992); they are similarly labeled with biotin-dUTP and PCNA and they change in number, shape, and distribution in much the same way (Hozák *et al.*, 1994b).

Although most replication takes place in factories, there is some extrafactory synthesis that increases as cells progress through S-phase (Hozák *et al.*, 1994b). There are special topological problems associated with replicating the last few base-pairs between two replicons (Sundin and Varshavsky, 1980, 1981) so it is attractive to suppose that the extrafactory labeling reflects a tidying-up duplication of hitherto unreplicated DNA.

B. Transcription Foci: RNA Polymerase II

Are many RNA polymerases similarly attached in large factories? Results using the light microscope—though not decisive—suggest that they might be (Jackson *et al.*, 1993; see also Wansink *et al.*, 1993). Encapsulated and permeabilized HeLa cells were incubated with Br-UTP; then 300–500 fluorescent foci were immunolabeled using an antibody againt Br-RNA (Fig. 7). The foci contain RNA polymerase II and Sm antigen, a component of the splicing apparatus. This ties the synthetic site into a solid-phase network that might play a role in post-transcriptional events like splicing and transport (e.g., Lawrence *et al.*, 1989; Carmo-Fonseca *et al.*, 1991; Carter *et al.*, 1993). Rough calculations again suggest each transcription focus contains ~50 active RNA polymerases and many templates. α-Amanitin, an inhibitor of RNA polymerase II, prevents incorporation into these foci. These foci remain after removing most chromatin as described in Fig. 3 (Jackson *et al.*, 1993), confirming that synthetic sites are attached to an underlying skeleton.

We might have expected tracking polymerases to be spread throughout open chromatin, but their apparent concentration into foci suggests they do not have the freedom to track everywhere. The simplest explanation of all these results is that many RNA polymerases are attached in factories like the DNA polymerases. However, this conclusion comes with a caution. Estimates of the numbers of RNA polymerases per focus are necessarily less exact than those for DNA polymerases, simply because we do not know the total number of elongating RNA polymerases or foci in a cell. Foci are so variable in size and intensity and close to the resolution afforded by light microscopy that it remains possible that many have intensities below the level of detection by fluorescence microscopy. Consider an extreme case in which ~25,000 polymerase II molecules (Cox, 1976) are in transcription units randomly distributed throughout euchromatin; this inevitably means

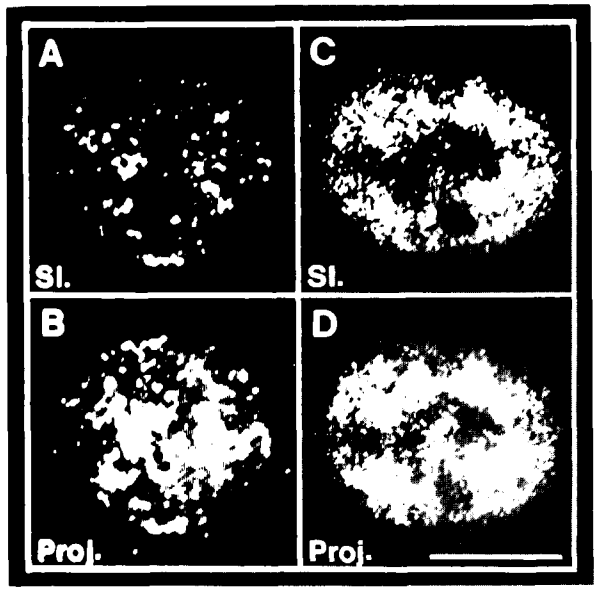

FIG. 7 [illegible] visualized by confocal microscopy. HeLa cells were permeabilized, incubated with Br-UTP for (A,B) 5 or (C,D) 10 min to extend nascent RNA chains by ~200 and [illegible] respectively, and sites containing Br-RNA indirectly immunolabeled. Nine optical [illegible] through a typical nucleus from each sample; A and C show a central [illegible] B and D the projections (Proj.) of the nine sections onto a single plane. Transcription [illegible] spread throughout nuclei, but concentrated in foci or factories. Bar [illegible] with permission of Oxford University Press.]

that there will be local variations in concentration, which—if above a critical threshold—will be detected. By judicious choice of threshold, number of polymerases per transcription unit, and volume occupied by nascent RNA, simulations show that nonrandom distributions can appear as apparently discrete foci (discussed by [illegible] *et al.*, 1993). Unfortunately, we have not yet been able to identify [illegible] by electron microscopy that might correspond to a polymerase II [illegible] by light microscopy. However, the nucleolus, which is [illegible] the skeleton described above, has a well characterized ultrastructure [illegible] provide us with a general model for the [illegible] of transcription [illegible]

C. [illegible]

[illegible] by light microscopy after incu-
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[illegible] and Br-UTP before immunolabeling any incorporated analogue; [illegible] discrete nucleolar foci are then visible (Jackson *et al.*, 1993). Again, these nucleolar foci remain after removing most chromatin as described in Fig. 3, confirming that synthetic sites are attached to an underlying structure.

Nucleoli have a well-defined ultrastructure. Several fibrillar centers, which equal the number of polymerase I foci described above, are each surrounded by a dense fibrillar component which is, in turn, embedded in the granular component. In which of these different nucleolar compartments transcription occurs has long been controversial (Jordan, 1991); several factors have contributed to this controversy, including the low resolution offered by autoradiography after incorporation of [^{3}H]uridine and the length of the labeling times required. We have recently resolved this controversy using permeabilized cells and a pulse of Br-UTP short enough to ensure that most transcripts remain at their site of synthesis. After immunogold labeling, gold particles were found over the dense fibrillar component (especially its border with the fibrillar center), indicating that the dense fibrillar component was the synthetic site (Hozák *et al.*, 1994a).

We imagine, then, that the nucleolus is built around fibrillar centers attached to the skeleton; the fibrillar centers store the polymerases, topoisomerases, and other proteins required for transcription. Polymerases directly organize this structure since its formation in mammalian cells is prevented by microinjecting antibodies to the enzyme (Benavente *et al.*, 1987) and yeast mutants with a deleted gene for the second largest subunit of the polymerase assemble several mininucleolar bodies rather than a normal crescent-shaped structure (Oakes *et al.*, 1993). About ~6 active cistrons (each ~5 μm long and packed with ~100 active polymerases) are associated with each fibrillar center in a human nucleolus (see Haaf *et al.*, 1991). Active polymerases—which resist elution in the experiment illustrated in Fig. 3 (Dickinson *et al.*, 1990)—lie on the surface of the fibrillar center and transcription occurs as a transcription unit slides end-on through them over the surface while the nascent rRNA is extruded into the dense fibrillar component. As a promoter emerges from one polymerase, it can soon engage another on the surface. On termination (i.e., when the 3′ end of the cistron has slid past a polymerase), the nascent transcript in the dense fibrillar component condenses into the granular component where it completes its maturation. Therefore the dense fibrillar component apparently slides over the surface of the fibrillar center, one end advancing while the other is converted into the granular component and newly inactive enzymes are recycled through the fibrillar center to the growing end of the dense fibrillar component.

This model can be reconciled with the beautiful images of spreads of ribosomal DNA (for a review, see Miller, 1981), if the hypotonic treatment used disperses the granular component and strips transcription units from

[illegible] the fibrillar center before spreading gives the well known [illegible]

[illegible] then attractive to suppose that attached RNA polymerase II molecules are also concentrated in analogous factories, with different templates sliding through polymerases on the surface; nascent transcripts would again be extruded through neighboring processing sites, perhaps then to pass along the skeleton to the cytoplasm (see also Lawrence *et al.*, 1989; Meier and Blobel, 1992).

IV. Replication and Transcription

The location of replication sites relative to transcription sites has been analyzed by incubating permeabilized cells from different stages of the cell cycle with both biotin-dUTP and Br-UTP (Hassan *et al.*, 1994; see also Wansink *et al.*, 1994). During G1 phase, there are ~300 transcription foci in a HeLa nucleus that aggregate on entry into S-phase into ~150 foci; these colocalize with sites of replication. Within about 30 min, many sites solely concerned in transcription reemerge, but the sites involved in replication remain transcriptionally active. Even late during S-phase—when deep heterochromatin has become duplicated—the replication sites remain transcriptionally active (Hassan *et al.*, 1994; but see Wansink *et al.*, 1994). This colocalization of sites of replication and transcription at the G1/S border suggests that transcription sites might seed assembly of replication factories (Hassan and Cook, 1994).

V. Repair Replication

The repair of damage [illegible] DNA by ultraviolet (UV) light involves excision of damage and [illegible] methods to fill the gap. Therefore sites where this repair takes place [illegible] immunolabeled after incorporation of biotin-dUTP, again not [illegible] spread but concentrated in discrete foci at sites that did not [illegible] DNA distribution (Jackson *et al.*, 1994a, b). The pattern [illegible] mutually intense repair took place at transcriptionally [illegible] transcription became inhibited by the effect of UV [illegible] at sites with little transcription. Repair with [illegible] absence of transcription, showing that the [illegible] coupled. Repair sites generally contained [illegible] cell nuclear antigen but not the tumor [illegible]

The repair activity seems not to be as closely associated with the skeleton as the S phase activity; after treatment with an endonuclease as in Fig. 3, 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*.

VI. Attachment Sites in a Model Loop

It is formally possible that many polymerases could track around a cluster of loops and so resist elution in the experiment illustrated in Fig. 3, if loops were too small to be cut. However, experiments with a model loop confirm that active polymerases resist elution even when the chromatin fiber is shredded (Jackson and Cook, 1993). They also allowed us to define the molecular ties at the bases of the loops.

A plasmid that possessed the SV_{40} origin of replication plus two promoters (i.e., the SV_{40}-early and human α1-globin promoters) was transfected into cos7 cells. There it replicated over 2–3 days to give many thousands of minichromosomes. About 1200 minichromosomes per cell resisted elution (i.e., were attached) while any extra copies that were transcriptionally and replicationally inactive could be eluted; this suggests that there are a saturable number of binding sites available to the plasmid. After cutting each attached minichromosome into ~400-bp fragments, ~90% of the contour length could be eluted; the remaining fragment or two were associated with a promoter or an engaged polymerase that was still able to run on along the remaining attached fragment.

These results were surprising and give us a very dynamic view of loop structure. Active minichromosomes might have been attached through a common sequence like an origin of replication or a SAR, but no single sequence was uniquely responsible for attachment; rather different parts of each minichromosome were attached at one or, at most, two points. Moreover, even though both transcription units were highly active, they only contained one polymerase. So we imagine that inert minichromosomes attach to a factory to become active, initially at one of the two promoters. Then a polymerase engages and the minichromosome becomes attached solely through the sequence at the polymerizing site. Finally the template dissociates, but its proximity to binding sites in the factory will mean that it can compete effectively with other minichromosomes for those binding sites. Therefore, at any moment individual templates will be at different

[illegible] no one point is always attached and different points [illegible] probabilities of attachment.

VII. Mitotic Chromosomes

The central structural feature of the interphase chromosome is then the chromatin loop, which is tied at its base through an engaged RNA polymerase (or promoter). Does this basic structural feature persist through the gross rearrangements that occur during mitosis? It seems that it does: the contour length of the loops—measured in a physiological buffer—remains unchanged (Jackson *et al.*, 1990) and both RNA polymerases I and II remain bound quantitatively (Matsui *et al.*, 1979), even though nascent transcripts abort (Shermoen and O'Farrell, 1991).

Nucleolar factories may also provide a detailed model for the structural reorganization of mitosis. It is known that as HEp-2 cells enter mitosis, the huge stores of the polymerase I transcription factor, UBF, remain bound at 6–8 of the 10 nucleolar organizing regions (NORs). Subsequently these local concentrations are symmetrically partitioned among daughter cells (Roussel *et al.*, 1993). Here the remnants of the interphase factory—the fibrillar center and its surroundings—persist into metaphase as visible entities, the NORs. However, most nucleolar material—especially the maturing transcripts—are lost from the NORs to form a dense mass of proteins and RNA that coats the surface of the mitotic chromosome (Hernandez-Verdun and Gautier, 1994). It is then attractive to suppose that analogous RNA polymerase II and III factories collapse onto each other to form the axial chromomeres of prophase; subsequent condensation of differently sized loops onto those factories would generate the bands typical of metaphase (Craig and Bickmore, 1993; Cook, 1994). Note that if the vestiges of these factories are segregated symmetrically to daughter cells like UBF, attachments involving RNA polymerases will be inherited by those daughters; as a result, the activity of particular genes will also be inherited (Cook, 1973, 1974).

Like the lamin exoskeleton, the lamin containing endoskeleton would also depolymerize during mitosis to allow chromosome segregation. On entry into G1 phase, it would repolymerize between factories to provide structural—and so functional—contiguity between factories and perhaps a solid phase for transcript movement. This nucleoskeleton would then play an essential role in integrating nuclear space, in much the same way that cytoplasmic intermediate filaments integrate cytoplasmic space (Lazarides, 1980).

VIII. Relationship to Studies on Other Structures

As the history of subnuclear structures seems to be a history of artifacts, it is foolish to claim that any preparation is free of artifact. However, the attachments of the chromatin fiber to the substructure seen under physiological conditions involve active polymerases that retain the activity that they had *in vivo;* if they had been generated during isolation, we would expect them to lose activity. How, then, do the studies described above relate to those involving other subnuclear structures that have led to different conclusions?

We believe that many of the differences result from the generation of new attachments during isolation. For example, if encapsulated cells are lysed, treated in the ways commonly used to isolate the substructures before returning them to a physiological buffer, and then the loop size measured, we find that different procedures give strikingly different results (Jackson *et al.*, 1990). Exposure to the mild hypotonic conditions generally used to isolate nuclei—usually the first step in the isolation of the substructures—roughly halves loop size, the precise value depending on the detailed ionic constitution of the isolation buffer. This reduction in loop size is not reversed by restoring the tonicity to normal and means that for every attachment existing *in vivo,* about one new attachment is created *in vitro* as nuclei are prepared. High concentrations of magnesium ions also irreversibly fix the chromatin into small loops (i.e., create new attachments). (Note that magnesium ions are chelated with an equal concentration of triphosphate in our physiological buffers.) Subsequent exposure to 2 *M* NaCl, like that used to prepare nuclear matrices, increases loop size from this lower value. Another popular procedure uses the detergent lithium diiodosalicylate (LIS) to generate scaffolds (Mirkovitch *et al.*, 1984). Lysis first increases loop size to ~100 kb, before a stabilization step reduces it to 15 kb. So here, five of six attachments are probably created by the procedure! As so many attachments are being created and destroyed during the isolation of matrices and scaffolds, it is then very difficult to be certain which of the ones studied *in vitro* actually preexisted *in vivo.*

Nucleoids are isolated directly from cells by extraction in 2 *M* NaCl, which increases loop size to 123 kb, so they probably lose only a few attachments. Therefore, their domain structure is very like that found using physiological conditions, with replicating and transcribed sequences (especially promoters and enhancers) being the most closely associated with the substructure (Jackson *et al.*, 1984). It is also reassuring that two completely different sets of conditions (i.e., direct lysis in 2 *M* NaCl to give nucleoids and lysis in physiological buffers) yield structures that have loops of roughly the same size, tied at their bases to the same skeletal structure through engaged RNA polymerases or promoters/enhancers, with active DNA and RNA polymerases attached to the skeleton and that the harsher treatment leads to slightly fewer attachments.

The finding of a lamin endoskeleton is consistent with observations of a distribution only at the periphery, if the dense chromatin generally prevents antibody access to the interior. Note that lamins are frequently associated with the interior of matrices (e.g., Capco *et al.*, 1982; Staufenbiel and Deppert, 1982; Fey *et al.*, 1984; Ludérus *et al.*, 1992) which, like the diffuse nucleoskeleton seen under physiological conditions, contains ribonucleoproteins and, perhaps, RNA (e.g., Long *et al.*, 1979; Fey *et al.*, 1986). It is usually assumed that this internal matrix cannot contain any lamins, and this may be the reason that attempts to isolate it and determine its structure have been so unsuccessful (Jack and Eggert, 1992); however, this failure is easily explicable if the assumption is incorrect. Intermediate filaments (which include the lamins) would then play a role in integrating both cytoplasmic and nuclear space. As lamins also bind DNA and/or chromatin (e.g., Burke, 1990; Glass and Gerace, 1990; Shoeman and Traub, 1990; Höger *et al.*, 1991; Yuan *et al.*, 1991; Glass *et al.*, 1993) and have been implicated in replication (e.g., Jenkins *et al.*, 1993), these results provide a physical basis for additional lamin functions within nuclei (e.g., Traub and Shoeman, 1994).

IX. Conclusions

The results described above suggest that the chromatin loop—the basic structural component of the interphase chromosome—is attached through polymerases and promoters to an underlying lamin-containing endoskeleton that ramifies throughout the nuclear interior. Both RNA and DNA polymerases are attached, either directly or indirectly through a factory, to this skeleton. As a result, loops are dynamic and polymerases static: points of attachment of loops continually change as templates are reeled through the fixed polymerases.

In HeLa cells, replication begins in several hundred small factories with diameters of 100–200 nm. As cells progress through S-phase many of the factories probably become inactive and are quickly dismantled, while others grow and fuse to give the large factories with long axes of 600 nm or more seen late during S-phase. These factories correspond to a subset of the dense structures known to electron microscopists as nuclear bodies.

Transcription of ribosomal genes also occurs in dense nucleolar factories in regions known as the dense fibrillar component surrounding the fibrillar centers. Although analogous factories involving RNA polymerases II and

III have not yet been visualized at the ultrastructural level, although the focal sites of transcription can be seen by light microscopy stereo-time that they too, will be concentrated within factories.

Immobilization of polymerases forces us to redraw our models for replication and transcription (Cook, 1989, 1991, 1993; Hozak *et al.*, 1995a). It also suggests new ways in which these processes might be controlled, for example at the levels of factory assembly and attachment of the chromatin fiber to the factory.

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