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On the structure of replication and transcription factories

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SUMMARY

Recent experiments suggest that active polymerases are concentrated in large structures, 'factories', within eukaryotic nuclei. Data concerning the structure of these factories is reviewed.

Key words: nucleoskeleton, replication, transcription, repair, chromatin loop

INTRODUCTION

Compared to the compartmentation found within the cytoplasm, the nucleus has traditionally been viewed as relatively unstructured. However, recent experiments suggest that it also has several different skeletons and functional sub-compartments. For example, the major nuclear processes of replication and transcription take place in specific structures, or 'factories', where many polymerases act in concert. We discuss here preliminary data on the structure of such 'factories'.

An appreciation of the role played by such compartmentation leads to a re-evaluation of how DNA and RNA polymerases work. (The term 'polymerase' is used here to describe the cluster of many different polypeptides that form an active complex in which the polymerizing sub-units are present only as minor components.) The traditional view involved enzymes that tracked along templates. This was sensible if the polymerases were small relative to the templates and if they acted alone. However, the immobilization of many polymerases within one factory means that the templates must move instead (Cook, 1989, 1991). Then the position of a gene in three-dimensional space relative to a factory will dictate how easily initiation of replication and transcription can occur.

ARTIFACTS

Isotonic salt concentrations are not usually used during nuclear fractionation or polymerase assay because they cause chromatin to aggregate into an unworkable mess, so more tractable conditions are used. However, these affect chromatin structure. For example, one-tenth the physiological salt concentration, often used to isolate nuclei and chromatin, destroys the 30 nm fibre and extracts a quarter of the DNA polymerases. Residual aggregation may be suppressed by adding 'stabilizing' cations like magnesium ions, but these generate (artifactual) attachments of the chromatin fibre to the sub-structure. Then it is not surprising that slightly different isolation pro-cedures generate different structures, each with its own characteristic set of sequences associated with a different sub-set of proteins. For example, **m**atrix-**a**ttached **r**egions or 'MARs' are bound to different proteins depending on the precise method of isolation, **s**caffold-**a**ttached **r**egions or 'SARs' are often specifically associated with topoisomerase II, and transcribed sequences are bound to 'cages'. Sceptics point to the fact that even those in the field cannot agree on which sequences are associated with which proteins in a particular sub-structure 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 compartmentation was only obtained with the use of more physiological conditions for biochemical studies or, better, by studying living cells.

'PHYSIOLOGICAL' CONDITIONS

More physiological conditions can be used during analysis if cells are first encapsulated in agarose microbeads (50-150 μm diameter). Agarose is permeable to small molecules so encapsulated cells continue to grow in standard tissue-culture media. When the cells 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 surrounding the nucleus (Fig. 1A-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 whilst 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 permeabilization. Note that almost all attachments to be discussed below involve active polymerases.

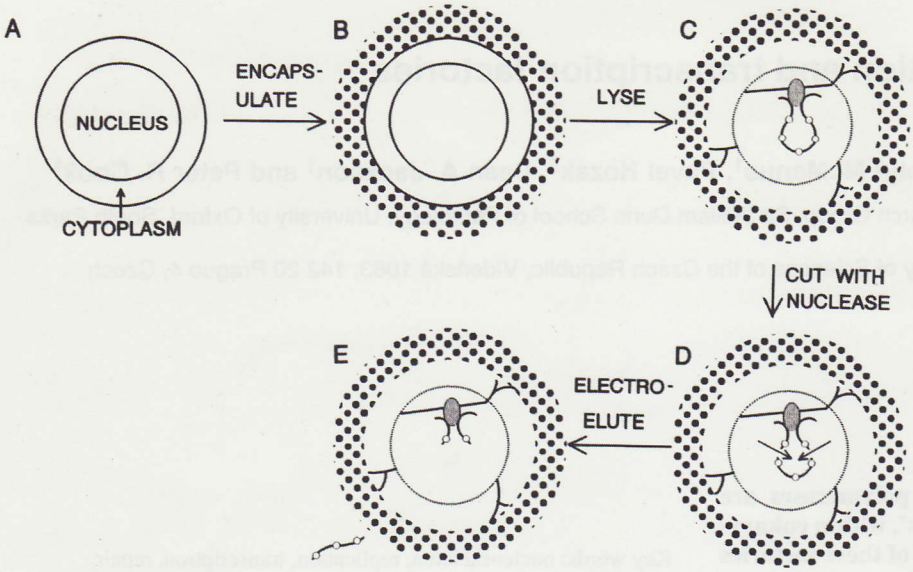

Fig. 1. Procedure for visualizing nucleoskeletons and associated structures. (A) Cells are (B) encapsulated in agarose (dots) and (C) lysed to leave a cytoskeleton, nuclear lamina (dotted circle) and nucleoskeleton (straight line) to which is attached a replication 'factory' (grey oval) and a DNA loop covered with nucleosomes (open circles). Dense chromatin obscures the nucleoskeleton but can be removed by (D) cutting the chromatin fibre with a restriction endonuclease, and then (E) removing any unattached fragments electrophoretically. Now that obscuring chromatin has been removed, the nucleoskeleton and associated factory can be seen in the electron microscope. (From Cook (1994) with permission of ICSU Press.)

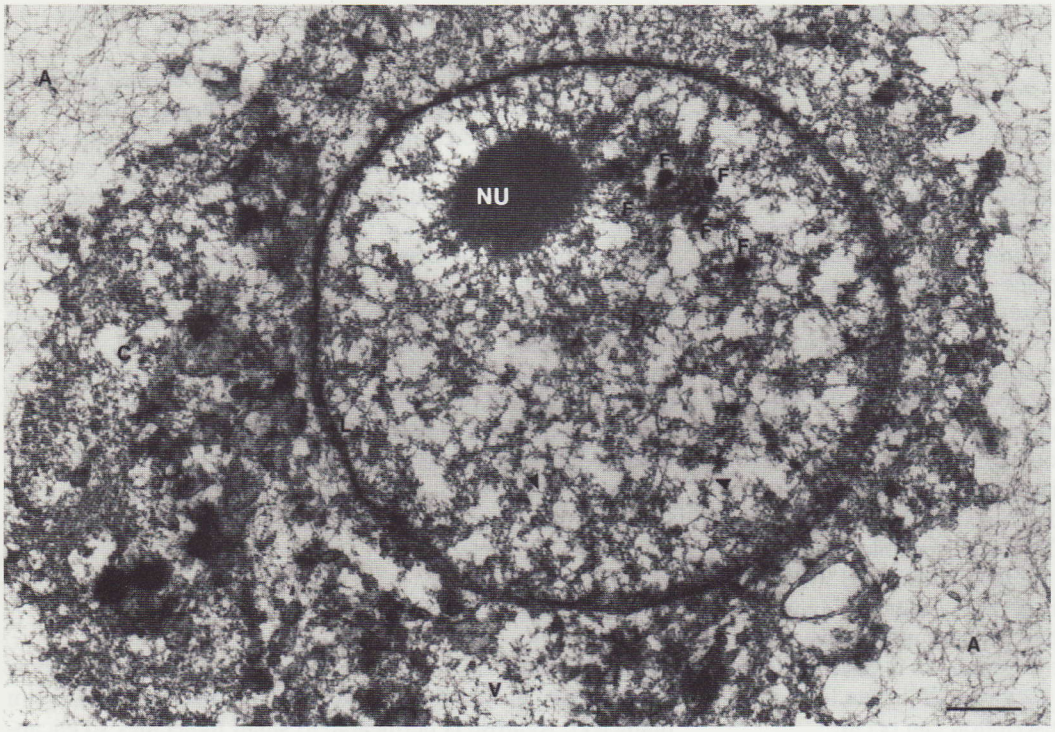

Fig. 2. Electron micrograph of cell 10 hours post-mitosis from which ~90% chromatin has been removed. Encapsulated cells were permeabilized, incubated with biotin-dUTP, treated with nucleases, chromatin eluted as in Fig. 1, incorporated biotin immunolabelled with 5 nm gold particles and a 500 nm resinless 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.)

The approach illustrated in Fig. 1 has been used to establish the basic structural features of interphase HeLa nuclei. For example, a nucleoskeleton was visualized by electron microscopy of thick sections; residual clumps of chromatin remain attached to a 'diffuse skeleton' that ramifies throughout the nucleus (Fig. 2). This network is morphologically complex, but its 'core filaments' have the axial repeat typical of the intermediate-filament family of proteins (Jackson and Cook, 1988) and its nodes can be immunolabelled (by both immunofluorescence and electron microscopy of thick sections) using various anti-lamin antibodies (Hozák et al., 1995). Electron microscopy of conventional thin sections also revealed lamin A in the interior as well as at the periphery. This was surprising as it is widely assumed that the nuclear lamins, as their name indicates, are confined to the nuclear periphery but these results suggest that lamins have been misnamed. (However, note that lamins have been found internally within nuclei, especially in certain pathological states, when mutated, or when over-expressed (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; Mirzayan et al., 1992; Bridger et al., 1993; Moir et al., 1994).

An average contour length of 86 kbp for the chromatin loops was deduced from the percentage of chromatin remaining attached to the skeleton and the size of the attached fragments.

As this length did not change during mitosis, the molecular machinery responsible for tying the chromatin into loops probably persists through mitosis (Jackson et al., 1990).

Active polymerases were also localized using the approach illustrated in Fig. 1; essentially all DNA and RNA polymerizing activity, as well as nascent DNA and RNA, resisted elution, suggesting that the enzymes were attached (either directly or indirectly) to the skeleton (Jackson and Cook, 1985b, 1986a,b; Jackson et al., 1988). Active enzymes cannot track around the loops, otherwise they would have been lost with the eluting chromatin.

REPLICATION FACTORIES

When living rat fibroblasts in S-phase are incubated with bromodeoxyuridine, ~150 foci containing the incorporated analogue can be immunolabelled (Nakamura et al., 1986). 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 heterochromatin is replicated (O'Keefe et al., 1992). Permeabilized mammalian cells (Fig. 1; Bravo and Macdonald-Bravo, 1987; Hozák et al., 1993) or demembranated frog sperm in egg extracts (e.g. Blow and Laskey, 1986, 1988; Hutchison et al., 1987, 1988; Mills et al., 1989) incorporate biotin-labelled dUTP into analogous foci, visualized in this case with fluorescently-labelled 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.

Synthetic sites can be immunolabelled with gold particles to a much higher resolution after incubating permeabilized cells with a sub-optimal 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 connected to it through an antibody bridge. In the first such experiments, most obscuring chromatin was removed as described in Fig. 1 before 400 nm thick (resinless) sections were viewed in the electron microscope (Hozák et al., 1993). 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. 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, the bodies seen by electron microscopy change in numbers, size, shape and distribution just like foci seen by light microscopy. They contain proteins specifically involved in synthesis (e.g. DNA polymerase α, PCNA, RP-A and DNA methyltransferase as well as others that might be involved in regulation (e.g. cyclin A, cdk2 and RPA70; Leonhardt et al., 1992; Adachi and Laemmli, 1992; Hozák et al., 1993; Cardoso et al., 1993; Sobczak-Thepot et al., 1993). They also correspond to a sub-set of the nuclear 'bodies' that have been seen over the years in conventional (thin) sections (Fig. 4; Brasch and Ochs, 1992; Hozák et al., 1994b). Electron micrographs of sections through these bodies suggest they are made of fine, tightly-packed, fibrils and that individual chromatin strands are attached at the surface.

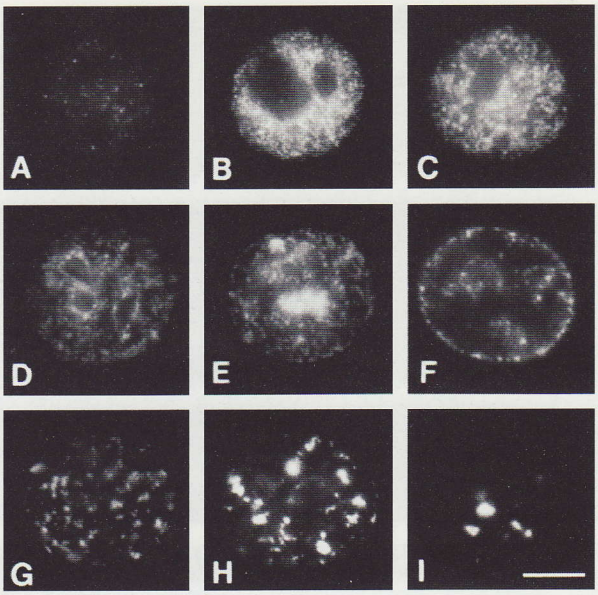

Fig. 3. Fluorescence micrographs of replication patterns found at different stages of S-phase. Synchronized HeLa cells were encapsulated in agarose, permeabilized, incubated with biotin-dUTP and incorporation sites indirectly immunolabelled. Fluorescence marks replication sites, which change in number and distribution as cells progress from (A) early to (I) late S-phase. Bar, 5 μm. (From Hozák et al. (1994a) with permission of the Company of Biologists Ltd.)

Simple calculations (based on the number of foci/nuclear bodies, the rate of fork progression, the spacing between forks, the size of the genome and the length of S-phase) imply that ~40 forks must be active in each early S-phase structure in a human cell. Therefore it seems appropriate to call them replication 'factories'.

Although most replication takes place in factories, there is some extra-factory 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 extra-factory labelling reflects a 'tidying-up' duplication of hitherto unreplicated DNA.

TRANSCRIPTION FACTORIES

Active RNA polymerases are also concentrated in factories (Jackson et al., 1993; Wansink et al., 1993). When encapsulated and permeabilized HeLa cells are incubated with Br-UTP, and then sites containing the incorporated analogue immunolabelled, 300-500 fluorescent foci can be seen. The foci also contain RNA polymerase II and rough calculations

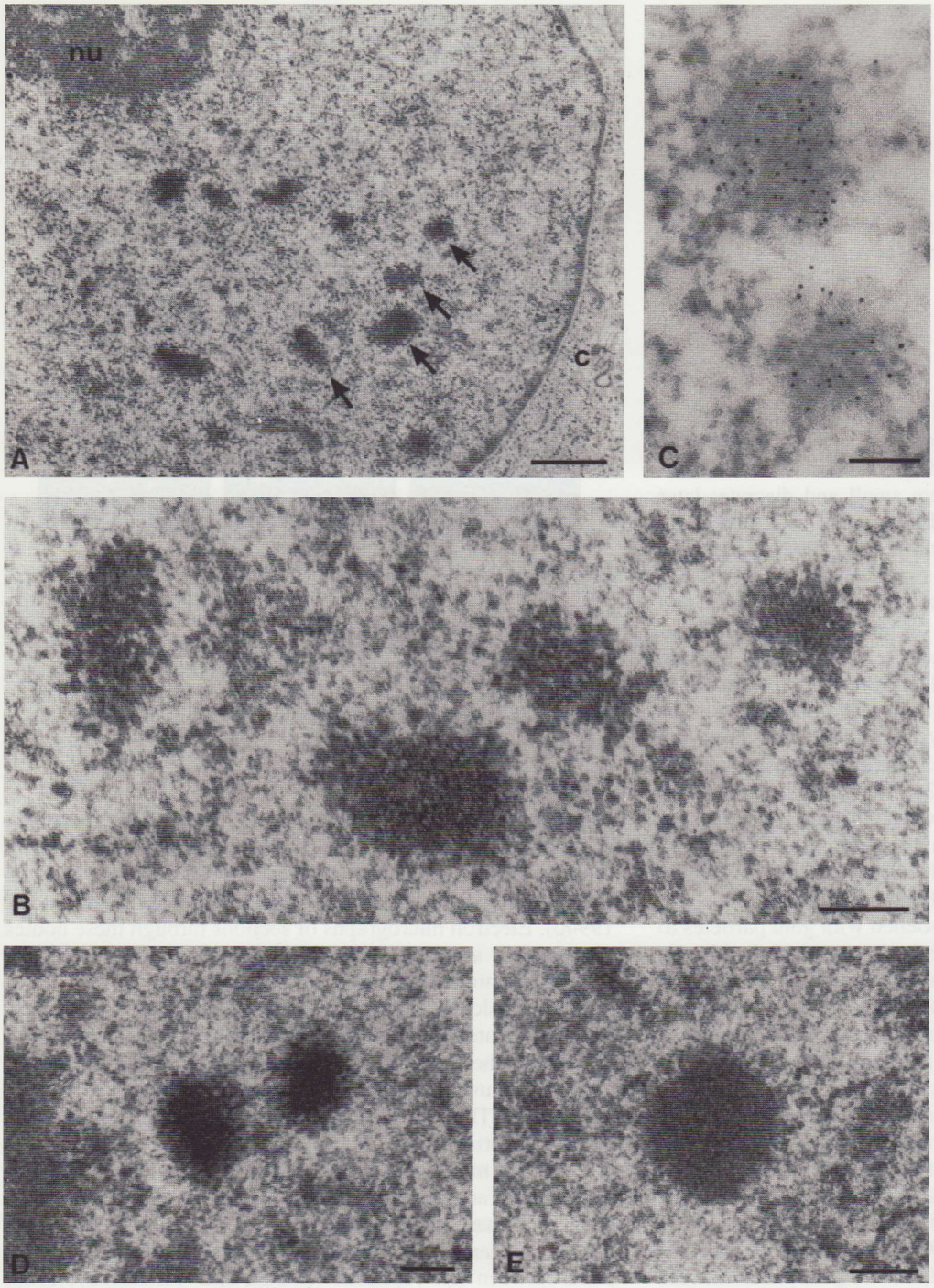

Fig. 4. The ultrastructure of replication factories/nuclear bodies in intact cells in mid S-phase. Synchronized cells were fixed without any further treatment and embedded in epoxy resin (A,B,D,E) or LR White (C). Epoxy resin preserves ultrastructure better; LR White is best for immunolabelling. (A) Low-power view showing clusters of factories (some are arrowed) typical of mid S-phase. c, cytoplasm. nu, nucleolus. Bar, 500 nm. (B) Arrowed factories in A. Factories consist of a dense fibrillar core from which radiates 20-25 nm fibrils. Different sections have different appearances; central sections (as D) reveal both zones, apical sections (as factory on left in B) show only profiles of radiating fibrils and intermediate sections have mixed profiles (as three factories on right in B). Bar, 200 nm. (C) Densely-staining region immunolabelled with anti-PCNA (5 nm gold particles); PCNA is found in replication factories. Bar, 100 nm. (D) Central sections through two factories. Bar, 200 nm. (E) Section through a dense factory that may have finished replicating. Bar, 200 nm. (From Hozák et al. (1994a) with permission of the Company of Biologists Ltd.)

again suggest each contains ~50 active RNA polymerases and many templates. These foci remain after removing most chromatin as described in Fig. 1 (Jackson et al., 1993), confirming that these synthetic sites are also attached to an underlying skeleton.

Nucleolar transcription factories are now relatively well characterized. Sites of nucleolar transcription can be seen by light microscopy after incubating permeabilized HeLa cells with α-amanitin (to inhibit RNA polymerase II) and Br-UTP, and immunolabelling any incorporated analogue; ~25 discrete nucleolar foci are then visible, and, again, these remain after most chromatin is removed (Jackson et al., 1993). In the electron microscope, nucleoli contain several 'fibrillar centres', which equal the number of polymerase I foci described above, surrounded by a 'dense fibrillar component' which is, in turn, embedded in the 'granular component'. Transcription (detected by immuno-gold labelling after incorporation of Br-UTP into nascent RNA) occurs in the dense fibrillar component on the surface of the fibrillar centre (Hozák et al., 1994a).

It then seems that the nucleolus is built around fibrillar centres attached to the skeleton; the fibrillar centres store the polymerases, topoisomerases and other proteins required for

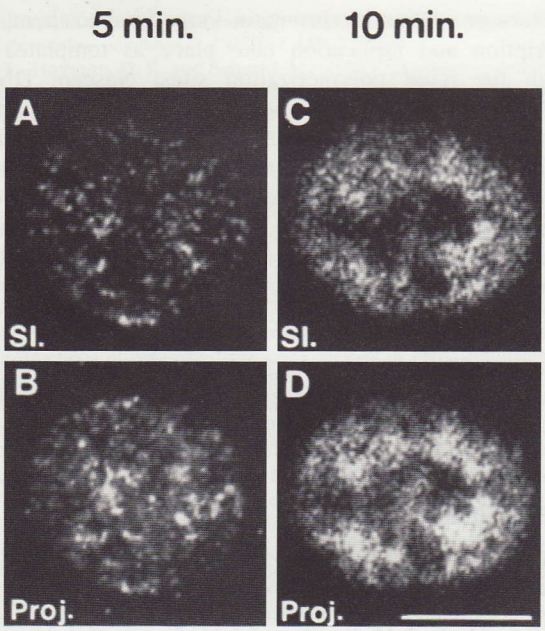

Fig. 5. Transcription sites visualized by 'confocal' microscopy. HeLa cells were permeabilized, incubated with Br-UTP for (A,B) 5 or (C,D) 10 minutes to extend nascent RNA chains by ~200 and ~400 nucleotides, respectively, and sites containing Br-RNA indirectly immunolabelled. Nine optical slices were taken through a typical nucleus from each sample; A and C show a central slice (Sl) and B and D the projections (Proj) of the nine sections on to a single plane. Transcription sites are concentrated in foci or 'factories'. Bar, 5 μm. (From Jackson et al. (1993) by permission of Oxford University Press.)

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). One, or a few, active cistrons (each ~5 μm long and packed with ~100 active polymerases) are associated with each fibrillar centre in a human nucleolus (see Haaf et al., 1991). Active polymerases, which resist elution in the experiment illustrated in Fig. 1 (Dickinson et al., 1990), lie on the surface of the fibrillar centre and transcription occurs as a transcription unit slides end-on through them over the surface whilst 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 centre, one end advancing whilst the other is converted into the granular component and newly-inactive enzymes are re-cycled through the fibrillar centre to the growing end of the dense fibrillar component (Hozák et al., 1994a).

There is also detailed information on the development of these factories. The total number of fibrillar centres is directly related to the rate of rRNA synthesis. For example, the ~234 in a fibroblast fall to ~156 on serum-starvation (Jordan and McGovern, 1981) and the few in a peripheral blood lymphocyte rise to ~100 as it is stimulated to divide (Hozák et al., 1989; Haaf et al., 1991). In other words, increasing transcription increases surface area and so the number of polymerases accessible to promoters. When nucleoli disassemble during mitosis, most nucleolar components disperse (Hernandez-Verdun and Gautier, 1994) but, remarkably, all polymerase I and most of the transcription factor, UBF, remain bound as the remnants of the centres aggregate into the nucleolar organizing regions on the chromosomes (Scheer and Rose, 1984; Roussel et al., 1993).

This provides us with a model for the structure of a nucleolar transcription factory: active polymerases lie on the surface of a storage core, in this case the fibrillar centre (Hozák et al., 1994a). It is then attractive to suppose that other active RNA and DNA polymerase molecules are also concentrated in analogous factories, with different templates sliding through polymerases on the surface as nascent nucleic acids are extruded.

REPLICATION AND TRANSCRIPTION

The relative locations of replication and 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 G_1 phase, the ~300 transcription foci in a HeLa nucleus aggregate on entry into S phase into ~150 foci; these colocalize with sites of replication. Within ~30 minutes, many sites solely engaged in transcription re-emerge, but the sites involved in replication remain transcriptionally active. Even late during S phase, when deep heterochromatin is being duplicated, the replication sites remain transcriptionally active. This colocalization of replication and transcription sites at the G_1/S border suggests that transcription sites seed assembly of replication factories (reviewed by Hassan and Cook, 1994). (Note also that sites where damage induced in DNA by ultra-violet light is repaired can colocalize with transcription sites; Jackson et al., 1994a,b.)

We can then imagine two extreme models to explain how replication factories evolve. Small (active) ones might grow progressively and/or aggregate into larger ones (Fig. 6, left). Such a model has the advantage of simplicity, as assembly of all factories could be triggered by only one event, and it fits the morphological observations that factories grow and/or fuse as cells progress through S-phase. Alternatively, factories of different sizes might be built ab initio at different stages in different parts of the genome (Fig. 6, right), but then some mechanism must control assembly of factories of the appropriate size at the right time and place. As it probably takes time to build a large factory, we would then expect to see many partly-built, but inactive, factories; however, very few are seen, consistent with the first alternative (Hozák et al., 1994b).

CONCLUSIONS

The results described above lead to the following model for the structure of factories. Inactive polymerases are stored in a central core built around a lamin-containing endoskeleton. Enzymes on the surface of this core only become active when

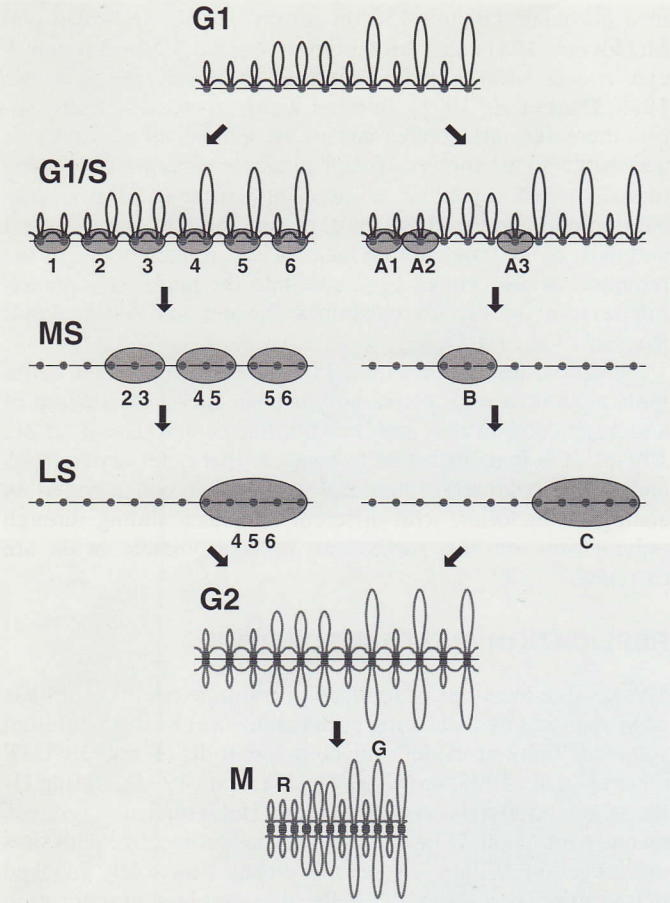

Fig. 6. Models for chromosome duplication. The G_1 chromatin fibre is shown looped by attachment to transcription factories (circles) on a skeleton (horizontal line). Small and large loops represent eu- and heterochromatin. For the sake of clarity, only one of the ~50 loops attached to each transcription factory during G_1, G_1/S and G_2, 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 G_2 fibre (shown as grey loops). On entry into mitosis (M), the skeleton disassembles (without changing the contour length of loops) and residual transcription factories (plus associated eu- 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 G_1/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. 4,5,6) 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 A1,2,3, 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. (1994a) with permission of the Company of Biologists Ltd.)

promoters or origins in chromatin loops bind to them. Then transcription and replication take place as templates slide through the fixed polymerization sites. Nascent DNA is quickly assembled into chromatin and extruded as part of a newly-replicated loop. In contrast, nascent RNA remains attached as it is extruded into adjacent processing sites on the skeleton. Transcription factories are also fundamental determinants of interphase structure as they contain the anchorage points of the chromatin loops and they seed the assembly of associated replication factories and processing sites.

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