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## ABSTRACT

Recent experiments suggest that the active polymerases involved in replication, transcription and the repair of damage in DNA are concentrated in large structures - 'factories' - within eukaryotic nuclei. This has forced us to reevaluate how polymerases work. The traditional view involved enzymes that tracked along templates and this was sensible if the polymerases were small relative to the templates and if they acted alone. However, immobilization of many polymerases within one factory means that the templates must move instead. Then the position of a gene in three-dimensional space relative to a factory will dictate how easily replication, transcription and repair can occur.

## Introduction

The cytoplasm is a complicated cellular compartment, with different regions dedicated to different functions. Various organelles and sub-compartments are organized around different skeletons and complex mechanisms direct molecular traffic between these sub-compartments and surrounding membranes. In contrast with this complexity, the nucleus has traditionally been viewed as relatively unstructured, with few major compartments (eg 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. We outline here the evidence for the localization of active polymerases in discrete structures that we will call factories'. Work using conditions close to the physiological and that maintain as much of the functional integrity as is conveniently possible will be emphasized.

The appreciation that active polymerases are concentrated in such factories' has led us to reevaluate how they 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 ( $9-10$ ). Then the position of a gene in three-dimensional space relative to a factory will dictate how easily the initiation of replication, transcription or repair occurs.

Artifacts
Isotonic salt concentrations are not generally used during nuclear fractionation or polymerase assay because they cause chromatin to aggregate into an unworkable mess; therefore biochemists have devised more tractable conditions, but these often affect chromatin structure. For example, one-tenth the physiological salt concentration is frequently used to isolate nuclei and chromatin but this generates (artifactual) attachments of the chromatin fibre to the substructure. Then it is not surprising that slightly different isolation procedures generate different structures, each with its own characteristic set of sequences associated with a different sub-set of proteins. For example, matrix-attached regions or 'MARs' are bound to different proteins depending on the precise method of isolation, scaffold-attached regions or 'SARs' are often specifically associated with topoisomerase II, and transcribed sequences are bound to 'cages'. Sceptics point to 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 $(8,26)$

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 $\mu \mathrm{m}$ 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 $(28,37)$ The agarose protects these cell remnants and, importantly, the encapsulated nucle 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 fiving 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 illustrates one approach we have used to visualize a nucleoskeleton within HeLa nuclei 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 (32) and its nodes can be immunolabelled (by both immunofluorescence and electron microscopy of thick sections) using various anti-lamin antibodies (22). 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 occasionally been found internally within nuclei (eg 15,6,43).]

In the experiment described in Fig. 1, the average contour length of a chromatin loops (ie 86 kbp ) was deduced from the percentage of chromatin remaining attached to the skeleton and the size of the attached fragments. This length did not change during mitosis, so the molecular ties holding the loops probably persist (33)

Active polymerases were also localized using a similar approach (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 directiy or indirectly) to the skeleton $(29,30,31,37)$. Active enzymes cannot track around the loops, otherwise they would have been lost with the eluting chromatin

A



C
$\longrightarrow$
Lyse
ulate
F


E


Thin
 Electro elute

D
Cut $\downarrow$


Fig. 1. A procedure for analyzing chromatin structure using 'physiological conditions.
(A) HeLa cells are (B) encapsulated in an agarose bead (dotted surroundings). (C) After permeabilization, the cytoskeleton, lamina, internal nucleoskeleton, associated transcription factory (oval) and DNA loop (line) covered with nucleosomes (circles) all become accessible to molecular probes. (D) Added endonucleases can now diffuse through the agarose and cut chromatin loops (arrows) so that ( $E$ ) most chromatin can be removed electrophoretically. (F) Skeletons, whether in the nucleus or cytoplasm, are best visualized by electron microscopy of thick sections. [From (12) with permission of the Company of Biologists Ltd.]

This procedure has been used to characterize:
(i) An internal lamin-containing nucleoskeleton, once obscuring chromatin is removed $(32,22)$.
(ii) The contour length of loops, from the average length and percentage of remaining DNA fragments (if fragment length is 8.6 kbp and $10 \%$ remains, contour length is $8.6 \times 1 /(10 / 100)=86 \mathrm{kbp})$. It does not change during mitosis, so the molecular ties holding loops persist (33)
(iii) Sequences remaining after elution; they are mainly promoters, enhancers and transcribed sequences, implying that engaged polymerases - which can still 'runtranscribed sequences, implying thate entaged ant to the skeleton (28-29, 34).
(iv) Replication sites; permeabilized cells (either before or after cutting and elution) are incubated with biotin-dUTP and sites containing the incorporated analogue indirectly immunolabelled with fluorescently-tagged antibodies (eg 20).
(v) Transcription sites; permeabilized cells (either before or after cutting and elution) are allowed to make RNA in the presence of Br-UTP, and then sites (ie factories) containing the incorporated analogue are immunolabelled using antibodies against Br-RNA $(35,50)$.
(vi) Repair sites; encapsulated cells in G1 phase are irradiated with ultra-violet light, incubated to allow repair to initiate, permeabilized and incubated with biotindUTP before repair patches containing the incorporated analogue are immunolabelled with fluorescently-tagged antibodies $(27,36)$


Fig. 2. Electron micrograph of cell 10 h post-mitosis from which $\sim 90 \%$ 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 nucleoskeleton that ramifies from nucleolus (NU) to lamina. Gold particles, which are not visible at this magnification, were concentrated in replication factories (F). Bar: $1 \mu \mathrm{~m}$. [From 20,21) with permission of the Company of Biologists Ltd.]

If DNA polymerases tracked along the template, we might expect sites of replication to be diffusely spread throughout euchromatin. It therefore came as a surprise to discover that active polymerases were not diffusely spread but concentrated within a few discrete foci. This was first demonstrated by incubating living rat fibroblasts in S-phase with bromodeoxyuridine; after the incorporated analogue was labelled with fluorescently-tagged antibodies, $\sim 150$ foci became visible (43). Early during S-phase the foci were small and discrete; later they became larger $(45,14,38,23,41)$ when heterochromatin is replicated (46) Permeabilized mammalian cells (Figs. 1 and $3,5,19$ ) or demembranated frog sperm n egg extracts (eg $2,3,24,25,42$ ) 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 (16). The foci remain even when most chromatin is removed $(45,19)$, implying that they are attached to the underlying skeleton.

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 (Fig. 2; 19). Electrondense 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 (eg DNA polymerase $\alpha$, PCNA, RP-A and DNA methyltransferase as well as others that might be involved in regulation (eg cyclin A, cdk2 and RPA70; 40-1-19-7-47). They also correspond to a sub-set of the nuclear 'bodies' that have been seen over the years in conventional (thin) sections $(4,21)$. 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.

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 $\sim 40$ 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 (21). There are special topological problems associated with replicating the last few base-pairs between two replicons (48-49) so it is attractive to suppose that the extra-factory labelling reflects a 'tidying-up' duplication of hitherto unreplicated DNA.


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: $5 \mu \mathrm{~m}$. [From 20, with permission of the Company of Biologists Ltd.]


Fig. 4. Transcription sites visualized by 'confocal' microscopy
HeLa cells were permeabilized, incubated with Br -UTP for ( $\mathrm{A}, \mathrm{B}$ ) 5 or (C,D) 10 min to extend nascent RNA chains by $\sim 200$ 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 (SI) 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: $5 \mu \mathrm{~m}$. [From 27 with permission of Oxford University Press.]

Active RNA polymerases are also concentrated in factories $(35,50)$. When encapsulated and permeabilized HeLa cells are incubated with Br-UTP and then sites containing the incorporated analogue immunolabelled, $\sim 500$ fluorescent foci can be seen (Figs. 1 and 4). We have recently visualized - using conditions in which all sites are detected --2000 such factories at the ultrastructural level; they contain three zones like the polymerase I factories discussed below: a region rich in RNA polymerase II and transcription factors, next to an area containing nascent transcripts which - in turn - abuts a region containing splicing components (unpublished results). Rough calculations again suggest that each must contain $\sim 15$ active RNA polymerases and many templates. They remain after removing most chromatin as described in Fig. 1 (35), confirming that they are also attached to the 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 $\alpha$-amanitin (to inhibit RNA polymerase II) and Br UTP, and immunolabelling any incorporated analogue; $\sim 25$ discrete nucleolar foci are then visible, and - again - these remain after most chromatin is removed (34) In the electron microscope, nucleoli contain several 'fibrillar centres' - which equal the number of polymerase I foci described above - surrounded by a 'dense tibrillar 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 (20).

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 transcription. One - or a few - active cistrons (each $\sim 5 \mu \mathrm{~m}$ long and packed with $\sim 100$ active polymerases) are associated with each fibrillar centre in a human nucleolus. Active polymerases - which resist elution in the experiment illustrated in Fig. 1 (13)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 polymerase on the surface. On termination (ie 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 (20). This provides us with a model for the structure of all transcription factories: active polymerases lie on the surface of a storage core, which lies next to a processing area.

## 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 $(18,50)$. During $G 1$ phase, the -300 transcription foci in a HeLa nucleus aggregate on entry into $S$ phase into $\sim 150$ foci; these colocalize with sites of replication. Within $\sim 30$ min, 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 G1/S border suggests that transcription sites seed assembly of replication factories (reviewed by 17).


Fig. 5. Visualization of sites of unscheduled DNA synthesis in (A-E) MRC-5 irradiated with different doses and ( $\mathrm{F}-\mathrm{J}$ ) HeLa cells at different times after irradiation.
A-E. Encapsulated cells in G1 were uv-irradiated with the doses indicated, grown for 1 h to allow repair to initiate, permeabilized with streptolysin and incubated with biotin-16-dUTP for 15 min ; sites containing incorporated biotin were then immunolabelled (goat-anti-biotin followed by a FITC-anti-goat antibody) before cells were photographed using similar exposures (except for that in A, for which the exposure was doubled).
F-J. As A-E, except cells were irradiated with $40 \mathrm{~J} / \mathrm{m}^{2}$ and grown for the times indicated. J illustrates DAPI-staining of cell in I. Bar: $5 \mu \mathrm{~m}$. [From (27) with permission of the Company of Biologists Ltd.]

Repair sites
The repair of damage induced in DNA by ultra-violet light involves excision of the damage and then repair synthesis to fill the gap. We have also visualized sites of repair synthesis in MRC-5 fibroblasts and HeLa cells in G1 phase (Fig. 1; 27,36 ). Encapsulated and permeabilized cells were irradiated with ultra-violet light and allowed to repair in the presence of biotin-16-dUTP; then sites containing the incorporated analogue were indirectly immunolabelled using a FITC-conjugated antibody. Again sites were not diffusely spread throughout nuclei but concentrated in discrete foci (Fig. 5). The pattern of repair foci changed with time; initially intense repair took place at transcriptionally-active sites but when transcription became inhibited it continued at sites with little transcription. Repair synthesis in vitro also occurred in the absence of transcription. Repair sites generally contained a high concentration of proliferating cell nuclear antigen but not the tumour-suppressor protein, p53.

The results show that the active polymases involved in all the major nuclear funtions are concentrated within discrete compartments or 'factories'. They also point to a central role for transcription factories in the organization of both the structure and the function of the nucleus. Active RNA polymerases and transcription factors in a factory tie the chromatin fibre into loops, and transcription takes place as templates slide through fixed polymerization sites in the factory. At the beginning of S-phase, these transcription factories seed the assembly of replication factories; then replication occurs as the template slides through the enalrged factory as nascent DNA is extruded into a newly-replicated loop. Moreover, when DNA is damaged, repair probably initiates within a transcription factory. Therefore, the position of a sequence relative to a factory is the key determinant of whether that sequence can be transcribed, replicated or repaired.

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