Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III

Ana Pombo, Dean A. Jackson, Michael Hollinshead, Zhengxin Wang, Robert G. Roeder and Peter R. Cook

Mammalian nuclei contain three different RNA polymerases defined by their characteristic locations and drug sensitivities; polymerase I is found in nucleoli, and polymerases II and III in the nucleoplasm. As nascent transcripts made by polymerases I and II are concentrated in discrete sites, the locations of those made by polymerase III were investigated. HeLa cells were lysed with saponin in an improved ‘physiological’ buffer that preserves transcriptional activity and nuclear ultrastructure; then, engaged polymerases were allowed to extend nascent transcripts in Br-UTP, before the resulting Br-RNA was immunolabelled indirectly with fluorochromes or gold particles. Biochemical analysis showed that ∼10 000 transcripts were being made by polymerase III at the moment of lysis, while confocal and electron microscopy showed that these transcripts were concentrated in only ∼2000 sites (diameter ∼40 nm). Therefore, each site contains approximately five active polymerases. These sites contain specific subunits of polymerase III, but not the hyperphosphorylated form of the largest subunit of polymerase II. The results indicate that the active forms of all three nuclear polymerases are concentrated in their own dedicated transcription sites or ‘factories’, suggesting that different regions of the nucleus specialize in the transcription of different types of gene.

Keywords: α-amanitin/RNA polymerase III/tagetitoxin/transcription

Introduction

Mammalian cells contain four different RNA polymerases defined by their characteristic locations and drug sensitivities (Chambon, 1974; Weinmann et al., 1975). A minor activity, which is resistant to α-amanitin, is concentrated in mitochondria, while the other three activities are all nuclear. Polymerase I (pol I) is nucleolar and resistant to α-amanitin but sensitive to low concentrations of actinomycin D; polymerase II (pol II) is nucleoplasmic and more resistant to actinomycin D but sensitive to low concentrations of α-amanitin; and polymerase III (pol III) is nucleoplasmic and inhibited by high concentrations of α-amanitin.

Nucleolar sites of pol I activity have been studied in detail (Shaw and Jordan, 1995). In electron micrographs, the nucleolus has a well-defined ultrastructure; pale fibrillar centres are surrounded by the dense fibrillar component, and both are embedded in a granular component. Active polymerases are found at the interface between the fibrillar centre and dense fibrillar component, nascent transcripts in the dense fibrillar component and maturing ribosomes in the surrounding granular component (e.g. Hozak et al., 1994). A nucleolus can be likened to an industrial park dedicated to the production of ribosomes; 45S rRNA is made in ‘factories’ on the surface of fibrillar centres and assembled into ribosomes in the granular component.

The finding that pol I is concentrated within a discrete nucleolar compartment begs the question: are active forms of pols II and III also concentrated in discrete, perhaps different, nucleoplasmic compartments? In principle, it should be a simple matter to answer this question, as various approaches are available to label transcription sites. In one, living or permeabilized cells are allowed to make RNA for a short period in the presence of modified precursors (e.g. [3H]U, Br-U, Br-UTP or biotin-CTP); then, incorporated analogues are detected by autoradiography or immunolabelling (Fakan et al., 1976; Jackson et al., 1993, 1998; Wansink et al., 1993; Hozak et al., 1994; Iborra et al., 1996). In a second, polymerases or their associated transcription factors are immunolocalized (e.g. Bregman et al., 1995; Grande et al., 1997; Kim et al., 1997; Pombo et al., 1998). In practice, several interrelated factors complicate analysis of pol III sites. First, the major pol III transcripts, i.e. 5S rRNA and the tRNAs, have lengths of only 120–121 and 75–95 nucleotides, respectively (Geiduschek and Tocchini-Valentini, 1988), and this short length inevitably makes detection difficult. Secondly, these short transcripts are made quickly, so precursors such as [3H]U and Br-U must be transported through membranes, converted into immediate precursors and equilibrated with internal pools before they can be incorporated into nascent RNA. While permeabilization allows direct access of precursors such as Br-UTP and biotin-CTP and control over elongation rates, the conditions used could disturb nuclear organization. Thirdly, the transcripts are both made and exported rapidly from synthetic sites; as a result, concentrations of transcripts detected by in situ hybridization (e.g. Matera and Ward, 1993; Matera et al., 1995) generally mark distant downstream sites. Fourthly, it is difficult to localize precisely the incorporated labels; after autoradiography, silver grains may be hundreds of nanometres away from incorporated [3H], and immunolabelling gold particles can lie 20 nm away from the Br-RNA or biotin-RNA they mark (Griffiths, 1993; Iborra and Cook, 1998). Fifthly, polymerases and their
transcription factors are often stored in certain parts of the nucleus, but used in others; as a result, the most intensely labelled sites are often inactive (e.g. Grande et al., 1997).

Despite these difficulties, Fakan and colleagues localized nucleoplasmic transcript sites after incubating cells with [3H]U for as little as 2 min; they found silver grains in autoradiographs over perichromatin fibrils at the edge of condensed chromatin (e.g. Fakan and Puvion, 1980; reviewed by Fakan, 1994). More recently, 500–8000 nucleoplasmic ‘foci’ or ‘factories’ where Br-UTP and biotin-CTP are incorporated have been visualized by immunofluorescence and high-resolution electron microscopy (Jackson et al., 1993; Wansink et al., 1993; Ibora et al., 1996, 1998; Fay et al., 1997). As labelling was sensitive to a low concentration of α-amanitin, these represent sites of pol II activity; however, no pol III sites were detected (Jackson et al., 1993; Wansink et al., 1993; Zeng et al., 1997). One study suggests that pol III sites are distinct from pol II sites; α-amanitin reduces the number of transcription sites detected in cells infected with adenovirus (Pombo et al., 1994).

We now characterize pol III sites in uninfected cells. Cells are lysed with saponin in a buffer that preserves transcriptional activity and nuclear ultrastructure; then, nascent transcripts are extended in Br-UTP, and the resulting Br-RNA is immunolabelled with fluorochromes or gold particles. In a HeLa cell, we find that ~10 000 transcripts are being made by pol III at any moment, and that these transcripts are concentrated in ~2000 sites with a diameter of ~40 nm. These sites are sensitive to high concentrations of α-amanitin that inhibit pol III, and contain pol III-specific subunits, but not the hyper-phosphorylated form of the largest subunit of pol II. Just as pol I is concentrated in a discrete (nucleolar) compartment, our results suggest that the active forms of pols II and III are also concentrated in their own dedicated ‘factories’ in the nucleoplasm.

Results

Establishing conditions for the detection of pol III transcripts

In order to visualize nascent pol III transcripts, we improved a procedure known to preserve considerable polymerizing activity and structure (Jackson et al., 1993). HeLa cells were encapsulated in agarose microbeads to protect them, and lysed with saponin in a ‘physiological’ buffer (PB). Then, [32P]UTP is incorporated efficiently into RNA (Figure 1A, curve 1). Addition of 100 mg/ml polyethylene glycol (not shown) or sucrose increases incorporation slightly (Figure 1A, curve 2). Addition of bovine serum albumin (BSA) (giving PB-BSA) further increases incorporation (Figure 1A, curve 3), but excess saponin then reduces it (Figure 1A, curve 4). Conditions giving maximum incorporation were used subsequently. Electron microscopy showed that these conditions preserved ultrastructure better than those used before (Figure 2). For example, electron density and fine structure in nuclei remain more like those seen in unlysed controls. Mitochondrial structure also survives, and probably underlies the preservation of transcriptional activity in this organelle (see below). These results show that gross activity and ultrastructure are well preserved under these conditions.

We next determined the maximum concentration of α-amanitin that inhibited pol II but left pol III unaffected. A typical experiment is illustrated in Figure 1B, and the results are summarized in Figure 1C and D. A
concentration of 0.2 μg/ml actinomycin D inhibits pol I (Figure 1C). Use of such a high concentration ensures that essentially all pol I is inhibited (see below), while incorporation into 5S and tRNA is reduced by only 7 and 4%, respectively (determined using autoradiographs like that shown in Figure 3A; not shown). Addition of α-amanitin progressively inhibits pol II, until concentrations between 0.1 and 2 μg/ml have no further effect (Figure 1C). Therefore, 2 μg/ml α-amanitin was used to inhibit pol II, and to leave pol III activity. This concentration is 15-fold higher than that required to inhibit pol II, but it has a negligible effect on pol III. Pol III activity was then abolished by 250 μg/ml α-amanitin, leaving the resistant mitochondrial activity (Figure 1D). This inhibition profile is like that seen with pure polymerases in vitro (e.g. Weinmann et al., 1975; Weil and Blatti, 1976).

The effects of these inhibitors are additive. Thus, 25 ± 4% (pol I) activity is inhibited by 0.2 μg/ml actinomycin (Figure 1C), and 27 ± 3% (pol I plus mitochondrial) activity resists 250 μg/ml α-amanitin (e.g. Figure 1B, curve 2). These percentages are similar to those seen previously in HeLa cells (e.g. McReynolds and Penman, 1974; Udvardy and Seifart, 1976; Weil and Blatti, 1976).

Number of engaged pol III complexes per cell
We next determined how many molecules of pol III are active at any moment in a HeLa nucleus. The number can be estimated if 5% of the total activity is due to pol III (Figure 1B and C) and a typical cell contains ~90 000 nascent transcripts (Jackson et al., 1998); then, ~4500 pol III complexes would be active (Figure 1). However, this crude estimate takes no account of differences in elongation rate, transcript length or rates of initiation and termination by the different polymerases. Therefore, we
estimated the number of pol III complexes more precisely using a strategy that does not rely on the use of α-amanitin and actinomycin. The approach depended on the shortness and defined length of the principal pol III transcripts, which enable them to be resolved by gel electrophoresis. Transcription reactions were carried out in the presence of Br-UTP, which has no detectable effect on pol III activity (see Materials and methods); therefore, the same conditions could be used for microscopy. [Similar results were obtained when UTP replaced Br-UTP (not shown).]

Nascent transcripts were extended for different periods in the presence of Br-UTP plus a trace of [32P]UTP, run on a gel and visualized by autoradiography (Figure 3A, lanes 1–5). The longest transcripts (made by pols I and II) either remained at the origin (zone A) or ran a short way into the gel (zones B and C). However, pol III transcripts ran well into the gel; completed 5S rRNA formed a sharp band of 120–121 nucleotides, and completed tRNAs a smear between 75 and 95 nucleotides. These assignments were confirmed using α-amanitin and actinomycin (not shown); for example, 200 μg/ml α-amanitin abolished all detectable labelling in the 5S rRNA band and tRNA smear (not shown). Reducing the concentration of Br-UTP from 100 to 1 μM reduced the amount of RNA made (Figure 3B).

Next, we investigated whether pol III reinitiated using two different inhibitors. Kovelman and Roeder (1990) used 0.05% sarkosyl to prevent reinitiation by the pure enzyme, but no 5S rRNA and tRNA bands could be detected in this complex system, despite increased incorporation by pols I and II (not shown). The second inhibitor, tagetitoxin (tagetin; Steinberg et al., 1990), proved more useful, even though its effects are complex. The extent of inhibition depends on nucleotide concentration, type of pol III transcription unit (Steinberg and Burgess, 1992) and enzyme purity (Kock and Cornelissen, 1991). However, the inhibitor efficiently stalls yeast pol III at pause sites on 5S and tRNA genes (Steinberg and Burgess, 1992), but allows pre-engaged mammalian polymerases to complete the first round of synthesis, while preventing subsequent rounds (Marais et al., 1994). Under efficient transcription conditions, an inhibitor that prevents recycling should allow a pre-engaged polymerase to complete synthesis of its transcript, but prevent synthesis of additional ones; under less efficient conditions, it would have little effect as so few first transcripts are completed.

We found that in 100 μM Br-UTP, tagetitoxin reduced incorporation in the 5S and tRNA regions (Figure 3A, compare lanes 4 with 6, and 5 with 7; Table I), but in 1 μM Br-UTP it had little effect (Table I). These results suggest that the drug limits synthesis to one round in 100 μM Br-UTP, but in 1 μM Br-UTP so few nucleotides are added that most polymerases do not complete synthesis of the first transcript. The number of transcripts completed by pol III can be calculated (see Materials and methods), as specific activities, cell numbers, loadings per lane and amount of label per transcript are all known. In 100 μM Br-UTP and tagetitoxin (i.e. under conditions where polymerases have ample time to complete the first transcript but cannot initiate new ones), ~1400 5S and ~6500 tRNA transcripts are made (Table I). As other transcripts are made by pol III (e.g. U6, MRP, 7SK, 7SL and RNase P RNAs), perhaps ~10 000 pol III complexes are active. In the absence of tagetitoxin, three-to-five times this number of transcripts are made, as new ones are initiated.

**Visualizing nascent transcripts made by the different polymerases**

We next visualized nascent RNA made by the different polymerases. Permeabilized cells were allowed to make RNA under conditions in which pols I and II extend nascent transcripts by ~1000 nucleotides, and each pol III completes three-to-five transcripts (Figure 3B; Table I); then, sites containing Br-RNA were immunolabelled indirectly with Cy3 and imaged in a ‘confocal’ microscope. In Figure 4A–H, single optical sections through the centre of nuclei illustrate distributions of nucleic acids (TOTO-3 fluorescence) and Br-RNA. In the absence of drugs, discrete foci containing Br-RNA are seen in nucleoli, the nucleoplasm and cytoplasm (Figure 4B). This pattern is generally similar to that seen previously (Jackson et al., 1993; Wansink et al., 1993; Iborra et al., 1996; Fay et al., 1997), except that these conditions preserve cytoplasmic (i.e. mitochondrial) foci, and >95% cells in the population have a similar appearance. [Using these improved conditions, variations in transcriptional activity of the type seen by Zeng et al. (1997) are not observed.]

If pol II is inhibited, the number of nucleoplasmic foci falls, to leave pol III foci (Figure 4D). Inhibiting both pols II and III abolishes all nucleoplasmic labelling, but leaves nucleolar and mitochondrial foci (Figure 4F). Actinomycin D affects the number and intensity of the various foci in different ways; for example, 0.05, 0.1 and 0.2 μg/ml actinomycin D progressively decreases the number of cells containing nucleolar foci to 20, 5 and 0% (not shown).

Modern ‘confocal’ microscopes have, at best, a resolution of ~200, ~200 and ~500 nm in the x-, y- and z-axes, respectively (Pawley, 1995). Two reasons make it difficult to count individual foci within sections such as those in Figure 4B. First, the faint foci seen could result either from low concentrations of Br-RNA lying in the plane of the section or from out-of-focus flare from high concentrations lying hundreds of nanometres above or below.

<table>
<thead>
<tr>
<th></th>
<th>1 μM Br-UTP</th>
<th>100 μM Br-UTP</th>
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<tr>
<td>5S rRNA</td>
<td></td>
<td></td>
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<tr>
<td>tRNA</td>
<td></td>
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<tr>
<td>Fraction total intensity (%)</td>
<td>0.41</td>
<td>0.52</td>
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<tr>
<td></td>
<td>2.7</td>
<td>2.3</td>
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<tr>
<td>Nucleotides × 10^6/ cell</td>
<td>0.010</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>2.3</td>
</tr>
<tr>
<td>Completed transcripts/cell</td>
<td>170</td>
<td>4900</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>30 000</td>
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<td>1200</td>
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Nascent transcripts were extended for 15 min in 1 or 100 μM Br-UTP ± 20 μM tagetitoxin and a trace of [32P]UTP, run on a gel, an autoradiograph prepared (as in Figure 3A), and the intensities in the 5S rRNA and tRNA zones expressed as a fraction of the total intensity in the lane. The number of nucleotides incorporated into these transcripts and the number of transcripts completed by each cell were then calculated (see Materials and methods).
Polymerase III factories

Fig. 4. Nascent pol III transcripts in (A–H) whole cells or (I–P) cryosections. Nascent transcripts were extended (100 μM Br-UTP; 15 min) with or without α-amanitin as indicated, sites containing Br-RNA were immunolabelled indirectly with Cy3 (nucleic acids were counterstained with TOTO-3), and red and far-red images of one optical section were collected using a ‘confocal’ microscope; pairs of images are shown without background subtraction (thresholding). (A–H) are views of individual cryosections of ~90 nm. Bar = 2.5 μm. (A and B) Foci containing Br-RNA are seen in nucleoli, the nucleoplasm and mitochondria. (C and D) Sufficient α-amanitin to inhibit pol II reduces the density of nucleoplasmic foci, without affecting nucleolar or mitochondrial foci. (E and F) A concentration of 250 μg/ml α-amanitin inhibits pols II and III, and abolishes nucleoplasmic labelling (but not nucleolar or mitochondrial foci). (G and H) Essentially no labelling is seen when Br-UTP is omitted. (I and J) Fewer, more discrete, Br-RNA foci of all types are seen in cryosections. (K and L) When pol II is inhibited, only a few pol III foci are seen in the nucleoplasm. (M and N) When pols II and III are inhibited, no nucleoplasmic foci are seen. (O and P) Essentially no labelling is seen when the primary antibody is omitted. Although resolution can be improved by deconvoluting information from serial sections, this brings attendant problems (e.g. Pawley, 1995). Secondly, many foci appear to merge one into another in the thick section, and these will be resolved better the thinner the section. Imaging (cryo)sections ~100 nm thick provides an alternative and direct way of improving z-axis resolution (Figure 4I–P); then, much more detail is seen in the TOTO-3 images (compare Figure 4A with I), and, most importantly, fewer, sharper, foci are seen (compare Figure 4B with J). [See Pombo et al. (1999) for a discussion of the improved resolution given by cryosectioning.] A concentration of 2 μg/ml α-amanitin reduces the number of nucleoplasmic foci to one-fifth (Figure 2D and L), leaving (pol III) foci which are about one-third as intense as the pol II foci (not shown). A concentration of 250 μg/ml α-amanitin eliminates all nucleoplasmic foci to leave only nucleolar and cytoplasmic (mitochondrial) foci (Figure 4F and N). No foci are seen in the nucleus or cytoplasm when Br-UTP or the primary antibody is omitted (Figure 4H and P), or after RNase treatment (1 mg/ml for 1 h at 37°C; not shown); therefore, they contain Br-RNA. These results are consistent with the nucleoplasm containing two populations of foci, with four-fifths being sensitive to 2 μg/ml α-amanitin (and so due to pol II), and one-fifth being resistant to 2 μg/ml α-amanitin but sensitive to 250 μg/ml (and so due to pol III).

As the true dimensions of sites <200 nm cannot be determined by light microscopy, and as transcription sites often lie within 200 nm of each other (Iborra et al., 1996), we imaged transcription sites by electron microscopy; Br-RNA in the cryosections was immuno-
2. 100 μM Br-UTP, 15 min
3. 100 μM Br-UTP, 15 min [1×]
4. 100 μM Br-UTP, 2 min [0.15×]
5. 5 μM Br-UTP, 15 min [0.1×]

Controls
5. As 1, plus 250 μg/ml α-amanitin
6. As 1, no Br-UTP
7. As 1, no anti-Br antibody
8. As 1, RNase treated (1 mg/ml; 2 h; 37°C)

Numbers of pol III sites
The total number of the different kinds of sites in a HeLa cell nucleus can be estimated (using standard stereological techniques; Materials and methods) from the densities in sections of known thicknesses, site diameter and nuclear volume. Therefore, we measured each in turn. Site numbers were counted in images obtained by confocal microscopy of whole cells or cryosections (not shown), and by electron microscopy of cryosections. The results obtained with the latter technique are given, as it provides the highest resolution and equivalent sensitivity. [Pombo et al. (1999) discuss the advantages and disadvantages of the different approaches, and show that they reveal exactly the same sites.] For this analysis, we define a cluster of gold particles as one containing more than one particle lying within 40 nm of another (centre–centre distance). [Approximately 40 nm is the maximum separation possible between two particles marking adjacent bromine residues in a transcript (i.e. roughly the length of two fully extended immunolabelling probes, each containing two immunoglobulins and a protein A molecule.)] In the absence of α-amanitin, there were 2.2 nucleoplasmic clusters/μm², which typically contained eight particles (Table II, row 1). Most of these clusters marked pol II + III sites, while a few (i.e. 0.2 clusters/μm²) with only two particles represented background (Table II, rows 5–8). In 2 μg/ml α-amanitin, there were only 0.55 clusters/μm² with six particles per cluster (Table II, row 2), against the same background.

Despite the sensitivity of the technique, it remained possible that some pol III sites went undetected, perhaps because they contained too little bromine. If so, increased bromine content should allow more undetected sites to be detected. However, varying the amount of incorporation over a 10-fold range, which was achieved using different Br-UTP concentrations and incorporation times, had only a marginal effect on cluster density (Table II, rows 2–4). These results are consistent with most pol III sites being detected.

Next, we determined the size of transcription sites using a method that allows the radii of spheres to be deduced from the radii of circular profiles seen in two-dimensional images of three-dimensional sections cut through the sample. Sites containing Br-RNA made by pols II + III and pol III had corrected radii of 23 ± 3 and 20 ± 2 nm, respectively (Figure 6).

We went on to calculate the number of sites in a typical nucleus from the site density (Table II), diameter (Figure 6) and nucleoplasmic volume (Materials and methods). Thus, 2.2 clusters/μm² marked nucleoplasmic sites containing Br-RNA made by pols II + III (Table II, row 1), of which 0.2 were due to background (Table II, row 6); the 2 clusters/μm² above background corresponds to ~9700 nucleoplasmic sites in a nucleus. Similarly, 0.35 clusters/μm² marked pol III sites (from Table II, rows 2 and 6), equivalent to 1800 sites per nucleus. As cells contain more active polymerizing complexes than sites, it follows that each site must contain more than one polymerase. Furthermore, as a pol III transcription unit of ~100 nucleotides is unlikely to be associated with more than one engaged polymerase (e.g. Braun et al., 1989; Bartholomew et al., 1993), it also follows that each site must contain more than one active transcription unit.

Pol III and its transcripts are found in dedicated sites
As each site contains more than one polymerase, are pols II and III active in the same site, or is each polymerase found in its own distinct site? If the different polymerases were intermingled within one site, and if most sites were detected, we would expect 2 μg/ml α-amanitin to reduce the intensity of labelling within a site without much effect.
Fig. 5. Transcription sites imaged by electron microscopy of cryosections. Nascent transcripts were extended (100 μM Br-UTP; 15 min) with or without 2 μg/ml α-amanitin; after fixation and cryosectioning, sites containing Br-RNA were immunolabelled indirectly with gold particles (5 nm), stained with uranyl acetate and imaged. Bars = 500 nm (A and B), 50 nm (C and D) and 75 nm (E). (A) Gold particles marking Br-RNA are concentrated in 102 nucleoplasmic clusters (small circles; each typically contains 11 particles). Five clusters (large circles) are also seen over the two nucleoli. Twenty-six and three lone particles lay over the nucleoplasm and nucleoli, respectively. (B) After extension in 2 μg/ml α-amanitin, nucleoplasmic labelling is concentrated in only four clusters (small circles; each typically contains eight particles); nucleolar clusters (large circles) are unaffected. Six and four lone particles lay over the nucleoplasm and nucleoli, respectively. (C–E) High-power views of typical clusters after extension without (C) and with 2 μg/ml α-amanitin (D and E); they have similar ultrastructures, but those in α-amanitin are usually marked by fewer particles.

on site density. On the other hand, if the two polymerases were concentrated in their own dedicated sites, inhibition of pol II activity should leave only the pol III sites. Results were consistent with this second possibility; site density was reduced to one-fifth (Figure 4; Table II).

We went on to see if pol II was not generally found
near pol III transcripts, nor pol III near pol II transcripts. Several factors complicate the analysis. First, nuclei contain pools of inactive polymerases far from transcription sites (e.g. Iborra et al., 1996). Fortunately, an antibody is available that detects the hyperphosphorylated form of the largest subunit of pol II (i.e. IIo; Bregman et al., 1995; Kim et al., 1997) that has been implicated in active transcription (Dahmus, 1996). Secondly, quantitative analysis of co-localization within sites only ~40 nm across is bedevilled by steric hindrance between probes with the true radius; any cut non-equatorially may give ‘polar caps’ that either appear smaller, or are missed because they are too small to be detected. The method allows corrections to be made so that the true size can be determined. Radii of >65 clusters of gold particles were measured and grouped together into bins (0–5 nm, 5–10 nm, etc.). Starting with the largest bin, the contribution of polar caps arising from spheres with this radius is calculated, and subtracted from the smaller categories; then, the next largest bin is treated in the same way, and so on. White rectangles reflect the contribution of polar caps, and black rectangles the corrected frequencies. Clusters marking RNA made by pols II + III and III had weighted mean radii of 23 ± 3 and 20 ± 2 nm, respectively; this difference was not significant (*p >0.05, Kolmogorov–Smirnov statistical test). For pol III, negative frequencies reflect caps arising from larger spheres that must have been present, but which went undetected.

Discussion

Improved conditions for transcription in permeabilized cells
Sites of nucleoplasmic transcription can be visualized after allowing living or permeabilized cells to extend nascent transcripts in the presence of various precursors (e.g. Br-U, Br-UTP or biotin-CTP), and then immunolabelling the incorporated analogues with fluorochromes or gold particles. The resulting Br-RNA proves not to be spread diffusely throughout euchromatin but is concentrated in discrete sites (see Introduction). As labelling is sensitive to a low concentration of α-amanitin, these mainly represent sites of pol II activity. However, sites of pol III activity have not been detected (Wansink et al., 1993; Zeng et al., 1997), except in cells infected with adenovirus (Pombo et al., 1994). We now describe conditions that preserve sufficient activity and ultrastructure to permit pol III sites to be visualized in uninfected cells.
HeLa cells are lysed with saponin in a ‘physiological’ buffer containing BSA; then, nascent transcripts are extended in Br-UTP, before the resulting Br-RNA is immunolabelled. Under these conditions, engaged polymerases extend nascent transcripts efficiently (Figure 1A), ultrastructure is preserved (Figure 2) and sites of activity of the four cellular polymerases can be visualized by both light and electron microscopy (Figures 4 and 5). In the absence of any drugs, discrete sites are seen in mitochondria (due to the mitochondrial polymerase), nucleoli (due to pol I) and the nucleoplasm (due to polys II and III).

The numbers of engaged pol III complexes

Several interrelated factors complicate any analysis of pol III activity, which is defined by its insensitivity to moderate concentrations of α-amanitin. First, nascent pol III transcripts are so short (i.e. <250 nucleotides) that they are difficult to label, are easily lost, and are completed so quickly that nascent transcripts exist only fleetingly. Secondly, pol III re-initiates in permeabilized cells, unlike polys I and II. Despite these difficulties, we attempted to estimate the numbers of active pol III complexes in a cell using an approach that relied on the short and discrete length of the major pol III transcripts. After extension in the presence of the inhibitor, tagetitoxin (tagetin), to prevent reinitiation, short pol III transcripts were freed from others by gel electrophoresis; then, we found that ~1400 5S rRNA and ~6500 tRNA transcripts were being made at any moment (Table I). [A typical (tetraploid) HeLa nucleus probably contains roughly this number of 5S and tRNA genes (e.g. Hatlen and Attardi, 1971; Marzluff et al., 1974; Sørensen and Frederiksen, 1991). Other genes transcribed by pol III include those encoding U6, MRP, 7SK and 7SL RNAs.] As 5S RNA and tRNA are the major transcripts made by pol III (Figure 3A), we conclude that ~10 000 pol III complexes are active at any time, within the range found previously (e.g. Marzluff et al., 1974).

The size of pol III sites

We determined the size of pol III sites by immunoelectron microscopy of cryosections. In such thin sections, antibodies have easy access to their targets, and pol III sites become marked by clusters of gold particles. These clusters had a radius of ~20 nm (Figure 6), which we assume is equivalent to that of the underlying complex containing Br-RNA. However, this complex cannot be much larger than the immunogold probe used for analysis, which has a radius of ~10 nm (i.e. a 5 nm gold particle coated by a layer of 3–6 nm of immunoglobulin; Lea and Gross, 1992; Iborra and Cook, 1998); therefore, sizes may be overestimated. A few clusters of clusters were seen that could house nascent RNA transcribed from the repeated genes in the major 5S rRNA locus (Figure 5E).

Numbers of sites

The density of nucleoplasmic sites seen here in two-dimensional images of cryosections is 1.7 times higher than densities seen before (Iborra et al., 1996). This probably results partly because many, if not all, pol III
sites were missed before, and partly because the improved approach allows some large sites to be resolved into two or more smaller ones (many clusters of clusters can be seen in Figure 5A). The total number of sites in three-dimensional space can be calculated (using standard stereological procedures) from the numbers and radii of clusters seen in two-dimensional sections (Table II, Figure 6); a typical nucleus contains ~9700 nucleoplasmic sites containing transcripts made by pols II + III, and ~1800 made by pol III.

How accurate is this estimate of the number of active pol III sites? Several reasons make it unlikely that many less active sites go undetected. First, increasing the amount of incorporation 10-fold should raise more of any undetected sites above the threshold of detection, but the increase was only 1.5 times (Table II). Secondly, we might also expect different detection methods to have different thresholds, but light microscopy of whole nuclei or cryosections, and electron microscopy of cryosections gave roughly similar results (Figures 4 and 5; not shown). Thirdly, any undetected sites can contain, in aggregate, only a tiny fraction of the total polymerizing activity. This follows because electron microscopy of cryosections is sufficiently sensitive to detect sites with one-twentieth the average Br-RNA content (in Figure 6, the volume of the smallest sphere detected is one-twentieth of the average), and so one-twentieth the average polymerizing activity. Even if ~10 000 nascent transcripts were housed in the ~1800 sites that are detected, plus another ~8200 that remain unseen, the latter could contribute, at most, only one-fifth of the total activity (because they have <1/20 the activity and four times the numbers). Therefore, it seems that most sites are detected. Note that in permeabilized cells, pol III transcripts completed in vitro seem unable to move away from the primary transcription site; instead, they accumulate at transcription sites, much like pol II transcripts (Iborra et al., 1996).

These results impose constraints on models of pol III transcription. If ~10 000 engaged polymerases are each housed in a distinct site, we have to suggest that there are two distinct populations that differ >20-fold in activity (see above), but there is no evidence for two such populations. However, all results are consistent with ~10 000 nascent pol III transcripts being contained in only ~1800 sites, so that each site would contain approximately five nascent transcripts. As a typical pol III transcription unit can probably accommodate only one polymerase (e.g. Braun et al., 1989; Bartholomew et al., 1993), each site would then be associated with approximately five templates. Occasional clusters of sites such as those seen in Figure 5E might accommodate 5S transcripts encoded by tandemly repeated genes.

Distinct transcription ‘factories’ containing pols I, II and III

As each site contains more than one polymerase, we investigated whether pols II and III were each active in their own dedicated sites using three different approaches. In the first, we compared site densities after inhibiting pol II. If the two polymerases were intermingled within a site and if most sites were detected, we would expect inhibition of pol II to have little effect on site density. However, site density fell to one-fifth (Figure 4; Table II), consistent with one-fifth of the transcripts being dedicated to pol III transcription. The second approach involved seeing if a polymerase was found next to transcripts made by the other enzyme. Although pols II and III often lay immediately next to their own transcripts (Figure 7A and B), they rarely lay next to those made by the other enzyme (not shown). However, such negative results are not decisive, especially when the approach is bedevilled by steric hindrance between immunolabelling probes. Convincing evidence against co-localization was obtained using a third approach that exploited this steric hindrance; cryosections were pre-incubated with an antibody to see if it blocked access of a second. We found that an anti-pol II antibody blocked access to pol II transcripts, but not to pol III protein or its transcripts (Figure 7C); conversely, an anti-pol III blocked access to pol III transcripts, but not to pol II protein or its transcripts (Figure 7D). Therefore, the results of all three approaches were consistent with pols II and III being active in spatially separate sites.

These results suggest that the two nucleoplasmic polymerases, like the nucleolar enzymes, are concentrated in their own dedicated sites. As each site probably contains a number of engaged polymerases, associated templates and transcripts, such sites have been christened transcription ‘factories’. Thus, each HeLa cell nucleus would contain a total of ~90 000 nascent transcripts, with ~15 000, ~65 000 and ~10 000 being made by pols I, II and III, respectively (Jackson et al., 1998; Table II). It remains to be seen to what extent nucleoplasmic factories resemble those found in nucleoli, but it is attractive to suppose that they share the same basic plan. The nucleolus possesses three major zones (Hozák et al., 1994; Shaw and Jordan, 1995): a fibrillar centre (a storage area containing pol I), a dense fibrillar component (where nascent transcripts are found) and a surrounding granular component (where primary transcripts are processed). According to this view, pol II factories would contain a store of enzymes, a zone containing approximately eight nascent transcripts and an adjacent processing area. Analogous pol III factories would be smaller, with only approximately five nascent transcripts. Indeed, the nucleoplasmic polymerases are often found immediately next to their transcripts (Figure 7A and B), which, in turn, are often next to sites rich in processing components (Pombo and Cook, 1996; Iborra et al., 1998).

Materials and methods

Buffers, saponin, BSA and inhibitors

‘Physiological buffer’ (PB) is 100 mM potassium acetate, 30 mM KC1, 10 mM Na2HPO4, 1 mM MgCl2, 1 mM Na2ATP (Sigma Grade I), 1 mM diethylthreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (pH 7.4). As the acidity of ATP batches varies, 100 mM KH2PO4 (usually 1/100th volume) can be added to adjust the pH. When triphosphates were added to PB, extra MgCl2 was added in an equimolar amount. PB* is PB plus human placental RNase inhibitor (10 U/ml; Amersham). PB-BSA and PB*-BSA contain 100 mg/ml BSA (Sigma A-7030; essentially free of fatty acids and γ-globulin). PBS + is phosphate-buffered saline (PBS) plus 1% BSA and 0.2% gelatin (from fish skin); where indicated, the pH was adjusted to 8.0. All buffers used up to fixation were ice-cold, unless stated otherwise.

Cells were lysed by addition of a large volume of freshly prepared saponin (Sigma S-7900 or S-4521). Saponin and BSA are mixtures of biomolecules and vary from batch to batch; saponin was titrated to give 95% lysis (assessed by immunofluorescence after Br-UTP incorporation; Figure 4) with each batch of BSA. Saponin (Sigma S-7900) was used for initial experiments (Figures 1, 2 and 4A–H), but a purer form (S-4521)
became available and was used at 0.25 mg/ml for the other figures and the tables. Both forms gave similar results, and higher concentrations of saponin are needed for lysis in BSA. Note that excess saponin can reduce transcriptional activity (e.g. Figure 1, curve 4) largely due to an effect on the proportion of activity due to pol III. In 5 and 100 μg/ml; Sigma) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP, 5 or 100 μM; Cappel) or tagetitoxin (tagetin; 20 μM; Cambio), cells were incubated for 10 min at 4°C, and then for (0.001–250 μM) and GTP.
1,000/1024 hybrid ‘confocal’ microscope (running under Comos 7.0a software) using a 0.7 nm aperture, the full dynamic range of grey scale in low scan/low signal mode, and Kalman filtration. Then, images were imported into Adobe Photoshop, ‘contrast-stretched’ and presented without further processing (i.e. without background subtraction). The volume of nucleoplasm (i.e. 660 ± 150 μm³; range 424–830; n = 10) in permeabilized HeLa cells was determined using Adobe Photoshop from serial optical sections collected at nominal 0.4 μm intervals through whole cells stained with TOTO-3. Each voxel had x and y dimensions of 0.17 μm and z dimension of 0.31 μm and not 0.4 μm (determined by reference to 10 μm latex Nile Red FluoroSpheres from Molecular Probes, as described by Visser et al., 1991).

Cryosections were prepared using a modification of existing methods (Tokuyasu, 1980; Griffiths et al., 1984; Tooze et al., 1991). During fixation in 8% paraformaldehyde, cells were scraped off the surface of the Petri dish and pelleted (200 g; 5 min); then, fixation was allowed to continue in the pellet for 20 min before it was dislodged. Next, the pellet was washed in PBS, transferred through three drops of 2.1 M sucrose in PBS over 2 h and then onto a copper block, frozen by immersion in liquid nitrogen, and stored in liquid nitrogen until use. Cryosections (90–150 nm thick, deducted from interference colour) were cut and captured on drops of 2.1 M sucrose in PBS. For light microscopy, sections were transferred to coverslips, quenched with glycin (as above), treated (2 min) with 0.1% Triton X-100 in PBS, washed, blocked, Br-RNA was immobilised, and images were collected (all as above, except the first antibody was used at 10 μg/ml). Use of a sheep anti-Br-DNA antibody (1/80; Biodesign International) led to the detection of 20% sites per nucleus (not shown).

For electron microscopy (Figure 2), fixed cells were post-fixed in reduced osmium tetroxide, contrasted, flat embedded in Epon (Tooze and Hollinshead, 1992) and imaged in a Zeiss EM 912 Omega transmission electron microscope equipped with both conventional, cooled slow-scan CCD (1024×1024 pixels; Proscan) and SIT 66 (Dage-MTI) cameras. For Figure 5, cryosections were cut from the same blocks used for light microscopy, transferred to grids instead of coverslips, quenched, treated with Triton, washed and blocked (all as above). Br-RNA was immunolabelled using the mouse anti-Br-Du antibody (10 μg/ml), washed (as above), incubated (2 h) with immunopurified rabbit anti-mouse IgG (10 μg/ml; Cappel), washed, incubated (30 min) with 0% normal goat serum (Jackson Laboratories), and (3–10 h) with goat anti-rabbit IgG conjugated with 5 nm gold particles (1/50; British BioCell) or protein A conjugated with 6 nm gold particles (1/100; prepared as described by Griffiths, 1993). (Two-layered sandwiches, i.e. the mouse anti-Br-Du antibody and goat anti-mouse conjugated with 5 nm gold particles (1/50; British BioCell), led to the detection of only 20% sites per nucleus (not shown).) Br-anti-Du antibody, rabbit anti-mouse IgG, biotinylated donkey anti-rabbit IgG (5 μg/ml; Jackson Laboratories, multiple-labelling grade) and goat anti-biotin conjugated with 5 nm gold particles (1/50; British BioCell), gave similar cluster densities but higher backgrounds (not shown.) Graps were washed five times over 2–8 h in PBS++, re-washed five times in PBS, fixed (10 min) in 0.5% glutaraldehyde in PBS, washed (three times) in water, incubated (4°C; 10 min) with 0.3% uranyl acetate in 2% methylcellulose, captured on wire loops, excess methylcellulose blotted on to a filter paper, recovered, and imaged in the electron microscope.

For Figure 7, cryosections were transferred to grids, quenched, treated with Triton, washed, blocked as above and labelled using three antibody layers in various ways. To label Br-RNA and pol II (Figure 7A), sections were incubated (2 h) with both mouse anti-Br-Du antibody (10 μg/ml) and the H5 antibody directed against the hyperphosphorylated form of the largest subunit of pol II (1/15 dilution of ‘nutridoma’ medium; Bregman et al., 1995). After washing, they were incubated (2 h) with both rabbit anti-mouse IgG Fc (γ-specific; 1/1500; Cappel) and biotinylated donkey anti-mouse IgM (μ-specific: 5 μg/ml; Jackson Laboratories), washed and incubated (30 min) with 10% normal goat serum (Jackson Laboratories), and (3–10 h) with goat anti-rabbit IgG conjugated with 5 nm gold particles (1/50; British BioCell) or protein A conjugated with 6 nm gold particles (1/100; prepared as described by Griffiths, 1993). [Two-layered sandwiches, i.e. the mouse anti-Br-Du antibody, rabbit anti-mouse IgG, biotinylated donkey anti-rabbit IgG (5 μg/ml; Jackson Laboratories, multiple-labelling grade) and goat anti-biotin conjugated with 5 nm gold particles (1/50; British BioCell), led to the detection of only 20% sites per nucleus (not shown).] Grids were washed five times over 2–8 h in PBS++, re-washed five times in PBS, fixed (10 min) in 0.5% glutaraldehyde in PBS, washed (three times) in water, incubated, (4°C; 10 min) with 0.3% uranyl acetate in 2% methylcellulose, captured on wire loops, excess methylcellulose blotted on to a filter paper, recovered, and imaged in the electron microscope.

For blocking experiments (Figure 7C and D), sections were incubated (2 h) with H5 or anti-RPC39, then (2 h) with H5, anti-RPC39 or mouse anti-Br-Du (all as above). When H5 was followed by anti-RPC39 (Figure 7C, line 1), cryosections were incubated successively with biotinylated donkey anti-rabbit IgG (2 h), normal goat serum (30 min) and goat anti-biotin conjugated with 5 nm gold particles (3–10 h). When anti-RPC39 was followed by H5 or anti-BrdU (Figure 7D), sections were incubated successively with biotinylated donkey anti-mouse IgG (2 h; 5 μg/ml; Jackson Laboratories), normal goat serum (30 min) and goat anti-biotin conjugated with 5 nm gold particles (3–10 h). In each case, no sites were detected when the antibody used to detect the target site (e.g. anti-RPC39 for Figure 7C, line 1) was omitted (not shown).

For quantitative analysis, a cluster of gold particles was defined as one containing more than one particle lying within 40 nm of another (centre–centre distance). The radius, r, of such clusters was determined after measurement (using SIS ‘Evision’ software supplied with the electron microscope) of the major, 2r, and minor, 2y (orthogonal), axes of each cluster, from r = √(x² + y²). The average diameter, D, of the underlying sites marked by these clusters was determined using the ‘sequential subtraction method’ (Weibel, 1979, 1980; Pombo et al., 1995). The numbers of sites in the three dimensions of a nucleus were calculated as follows (Weibel, 1979): (i) the density (nₛ) of clusters in two-dimensional images of three-dimensional sections was measured; (ii) the volume density (nᵥ) was calculated using Abercrombie’s formula nᵥ = nₛ(D + T), where T is the section thickness; and (iii) the number of sites per nucleus were calculated with knowledge of the nuclear volume. Calculated values in three-dimensional space are sensitive both to values of site radius and to nucleoplasmic volume.

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