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Active RNA polymerase I is fixed within the nucleus of HeLa cells

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We have investigated whether active RNA polymerase I, the enzyme responsible for transcribing ribosomal RNA, is immobilized by attachment to a large subnuclear structure in HeLa cells. As unphysiological salt concentrations induce artifacts, we have used isotonic conditions throughout the preparative and analytic procedures. Cells are encapsulated in agarose microbeads and lysed in Triton and a 'physiological' buffer; then soluble proteins and RNA diffuse out through the agarose pores to leave encapsulated chromatin. This can be manipulated without aggregation but is accessible to molecular probes; it retains the replicational and transcriptional activities of the living cell. After treatment with a restriction endonuclease, most chromatin can be removed from beads by electrophoresis: then active ribosomal genes and polymerase I remain behind. Active ribosomal genes are very accessible to nuclease digestion whilst the rest are even more inaccessible than inactive globin genes. Our observations confirm the complex organization of rDNA within nucleoli and are compatible with transcription occurring at fixed sites. A model for transcription involving an attached polymerase is presented.

Key words: domains/nucleoskeleton/nuclear matrix/ribosomal DNA/RNA polymerase I

Introduction

Ribosomal genes (rDNA) are clustered in human cells in tandem arrays on the short arms of five chromosome pairs (Henderson *et al.*, 1972; Wellauer and David, 1973). During interphase, these genes are organized into nucleoli, which are the site of rRNA synthesis and processing (Hadjiolov, 1985; Jordan, 1987). The nucleolus is a complicated structure containing RNA polymerase I, the enzyme that transcribes rDNA, as well as topoisomerase I, nucleolin and other proteins (Ochs *et al.*, 1983; Scheer and Rose, 1984; Guldner *et al.*, 1986; Benavente *et al.*, 1987; Ochs and Smetana, 1989). Pulse-labelling indicates that nascent RNA is associated with a dense fibrillar component (Fakan and Puvion, 1980; Wachtler *et al.*, 1989); RNA polymerase I and topoisomerase I are associated with the fibrillar centre (Scheer and Rose, 1984; Reimer *et al.*, 1987). Considerable structure survives hypertonic extraction but, paradoxically, little, if any, is preserved when cells are disrupted under hypotonic conditions during the preparation of 'Miller' spreads. Then rDNA associated with polymerase and transcript is seen, with no hint of any organizing framework (Miller and Beatty, 1969; Miller, 1984).

Images of 'Miller' spreads underpin current models of transcription: soluble RNA polymerases process along the template as they synthesize nascent RNA (Darnell *et al.*, 1986). In contrast, two studies suggest that active RNA polymerase II is not processive but fixed to a larger nuclear structure; transcribed genes are associated with nuclear 'cages' isolated in 2 M NaCl (reviewed by Jackson *et al.*, 1984a) and active polymerases remain associated with residual chromatin when most is removed from nuclei under more physiological conditions (Jackson and Cook, 1985a,b). We now test whether active RNA polymerase I is also attached to a larger nuclear structure.

As unphysiological salt concentrations might induce chromatin aggregation (reviewed by Cook, 1988; Jackson *et al.*, 1990), we use isotonic conditions throughout the preparative and analytic procedures. Living cells are encapsulated in agarose microbeads (Cook, 1984; Jackson and Cook, 1985a; Jackson *et al.*, 1988) and lysed with Triton X-100 in a 'physiological' buffer; then soluble proteins and RNA diffuse out through the pores to leave encapsulated chromatin. This can be manipulated freely without aggregation but is accessible to restriction enzymes. Whilst we cannot be certain that any *in vitro* preparation is free of artifact, this one contains intact DNA and most of the replicative and transcriptional activities of living cells (Jackson *et al.*, 1988). For example, under the appropriate conditions our preparation can replicate *in vitro* at 85% of the rate found *in vivo* in a cell-cycle dependent manner (Jackson and Cook, 1986; Jackson *et al.*, 1988). Unfortunately, as the absolute rate of transcription *in vivo* is not known, relative efficiencies *in vitro* cannot be estimated; however, *in vitro*, the transcription rate is more than seven times the replication rate and we know of no preparation that transcribes more efficiently (Jackson *et al.*, 1988).

Whether the polymerase is attached can be addressed by cutting encapsulated chromatin with a restriction endonuclease and then removing any unattached chromatin by electrophoresis. If the transcription complex is unattached, it should co-electroelute with chromatin fragments from the bead; if attached, it should remain behind. The 'physiological' buffer is present during lysis, nucleolytic digestion, electrophoresis and enzyme assay. Chromatin containing >150 kb DNA can electroelute from beads (Jackson and Cook, 1985a) and here it is cut into fragments of about 10 kb or less. In some cases, nascent RNA is also trimmed from the transcription complex prior to electroelution using RNase.

Results

Accessibility of ribosomal DNA

Our approach requires that some restriction sites in ribosomal chromatin can be cut, so that any detached fragments can be removed. Therefore we initially investigated accessibility

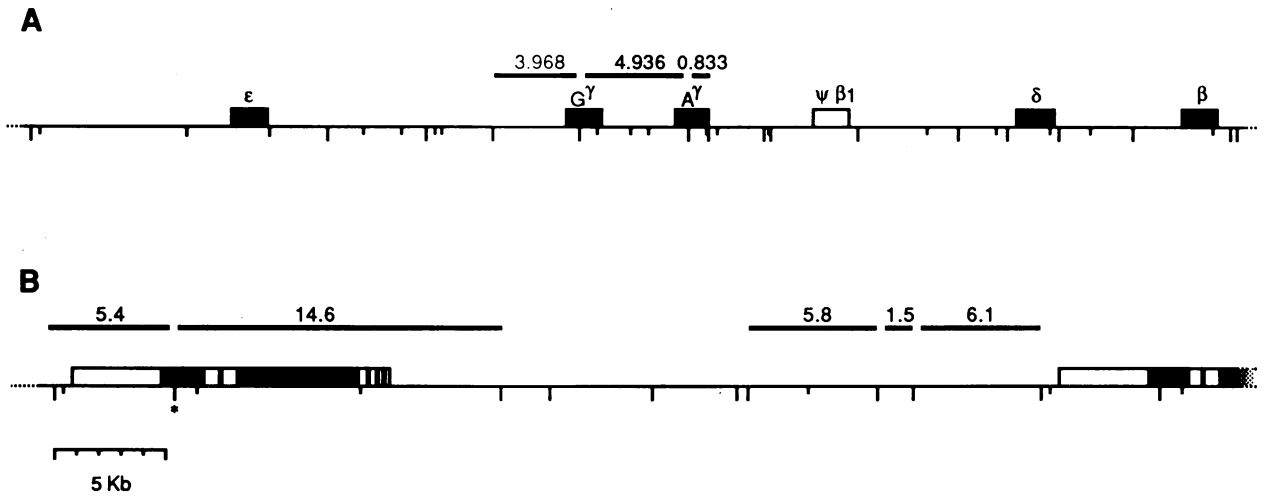

Fig. 1. Restriction map of (**A**) the human β-globin and (**B**) the rDNA loci. Long vertical line, *Pst*I sites; short vertical line, *Eco*RI sites. >50 *Hae*III sites scattered throughout the rDNA locus are not shown. The position and size (kb) of *Pst*I bands detected by (A) the 3.2 kb *Hin*dIII fragment from the globin locus and (B) probes from various parts of the rDNA locus used in Figure 3 are indicated by thick lines above the maps. (A) Globin genes are indicated by filled boxes, the pseudo-gene by the open box. (B) The 18,28 and 5.8S genes are indicated by filled boxes and the transcribed spacer by open boxes. *indicates a polymorphic site in HeLa (see McCallum and Maden, 1985; Safrany and Hidvegi, 1989), which gives rise to the two bands seen in Figure 3D.

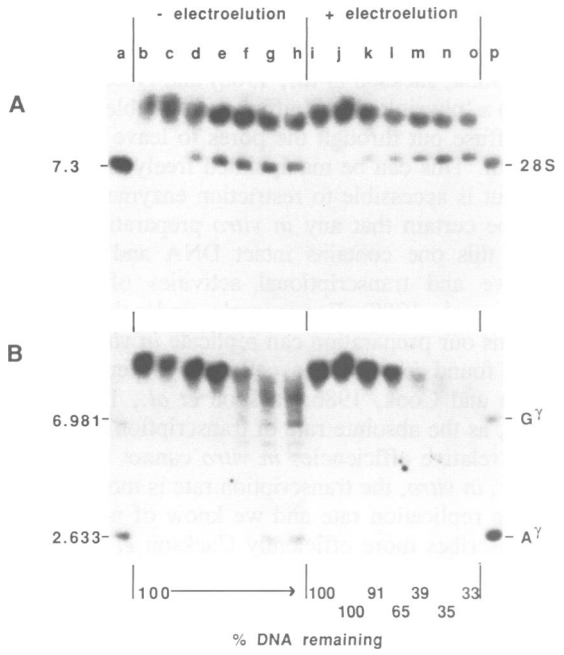

Fig. 2. rDNA is accessible and attached. HeLa cells were labelled with [^{3}H]thymidine, encapsulated (24 × 10^6 cells/ml), lysed and incubated with 0, 0.5, 5, 15, 50, 500 and 5000 units/ml *Eco*RI for 1 h at 32°C. Half of each sample was electroeluted (16 h, 4°C). DNA remaining in beads in both halves was freed of RNA and protein, subjected to electrophoresis, blotted and then the filter hybridized successively with the ribosomal (**A**) or β-globin (**B**) probe (i.e. the 7.3 kb *Eco*RI fragment or the 3.2 kb *Hin*dIII fragment). Finally autoradiographs were prepared. Tracks a and p: 5 μg HeLa DNA completely digested with *Eco*RI. Tracks b−h: samples digested with 0−5000 units/ml *Eco*RI, but not electroeluted (i.e. total DNA). Tracks i−o: samples digested with 0−5000 units/ml *Eco*RI and electroeluted (i.e. attached DNA).

of sites within the locus and, as a control, those of the non-transcribed β-globin genes. (Restriction maps of the two loci are given in Figure 1.) Encapsulated and lysed cells were incubated with varying amounts of *Eco*RI and then, without electroeluting any chromatin, DNA fragments were sized on a gel. The sizes of rDNA fragments were analysed by blotting and hybridization after applying equal numbers of beads to separate lanes (Figure 2A, left-hand side). Lane a illustrates fragments given by completely digesting naked DNA and lanes b−h those resulting from digesting chromatin of lysed cells. Some non-specific breakage occurs in the absence of *Eco*RI during sample preparation and the resulting large fragments contain rDNA and either remain in beads or run together as an unresolved band at the top of the gel (lane b). 5 units/ml *Eco*RI cut some ribosomal repeats into fragments containing 7.3 kb rDNA (lane d); addition of up to 5000 units/ml has little further effect (lanes e−h).

When the same filter was reprobed with a globin probe, small globin fragments were only detected after treatment with the highest concentrations of *Eco*RI (Figure 2B, lanes f−h). These results confirm the general finding that inactive genes are relatively inaccessible (Weisbrod, 1982) and that there are two types of ribosomal chromatin, with a fraction being accessible (Reeves, 1978; Franke *et al.*, 1979; Cech and Karrer, 1980; Borchsenius *et al.*, 1981; Davis *et al.*, 1983; Conconi *et al.*, 1989). Under our growth conditions the accessible rDNA genes within HeLa cells represent 25−35% of the total (Figure 2A, lanes e−h). Even at the lowest levels of digestion used here each repeat in this accessible fraction is cut at least twice. In most subsequent experiments levels of digestion were sufficient to cut all accessible sites within the ribosomal locus.

rDNA is attached

Chromatin fragments resisting electroelution (defined as attached fragments) were also analysed (Figure 2, right-hand side). Removal of 67% of total chromatin led to removal of little ribosomal chromatin; ~90% of the 7.3 kb fragment (arising from the accessible fraction) and ~50% of the genes that resist digestion remain in beads (Figure 2A, cf. lanes g/h and n/o). In contrast, essentially all globin chromatin was lost (Figure 2B, lane o). Although ribosomal and globin

chromatin are cut into fragments of roughly the same size, only the globin chromatin escapes; the ribosomal chromatin is attached. Essentially similar results for the two loci were obtained with *Pst*I, or using field-inversion conditions during electroelution (results not shown; see also Jackson,D.A., Dickinson,P. and Cook,P.R., submitted).

Quantitative analysis of Figure 2 might be complicated by the presence of partial-digestion products. Therefore we cut and electroeluted detached material as before, but then purified DNA and completely recut it with the same enzyme (Cook and Brazell, 1980). If sequences are attached, their concentration should be higher in the fraction resisting electroelution than in total DNA. A typical experiment is illustrated in Figure 3, where all but 18% of the chromatin was removed. In the 1× tracks, equal weights of total and attached DNA were applied to the gel. The concentration of globin fragments in the attached fraction was slightly less than that in the total fraction (cf. 1× tracks in A and B); these were readily lost and so were remote from attachment sites. In contrast, rDNA fragments containing the 18 and 28S genes were enriched >3× in the attached fraction (Figure 3C,D). If all remained, enrichments of 1/18% or 5.6× would be expected; the observed enrichments of 2.8−4.6× show 50−80% of these sequences to be attached, consistent with the results in Figure 2.

Parts of the rDNA locus are closer to attachment sites than others (or attached in a larger fraction of the repeats); for example, the 1.5 kbp fragment in the non-transcribed spacer behaves as if it is attached in all repeats and is more enriched than the adjacent fragments (Figure 3E,F). This enrichment highlights the lack of correlation between enrichment and fragment size: these fragments are about 1/100 the size of those we know can escape (Jackson and Cook, 1985b). Again, essentially similar results were obtained using *Eco*RI (both with HeLa or another human cell-line, K562) or field inversion conditions for electroelution (results not shown; see also Jackson,D.A., Dickinson,P. and Cook,P.R., submitted). It also seems improbable that nascent RNA makes the transcription complex and associated chromatin too bulky to electroelute as pretreatment with sufficient RNase to remove >95% nascent RNA has no effect on the enrichments found subsequently (results not shown; see below).

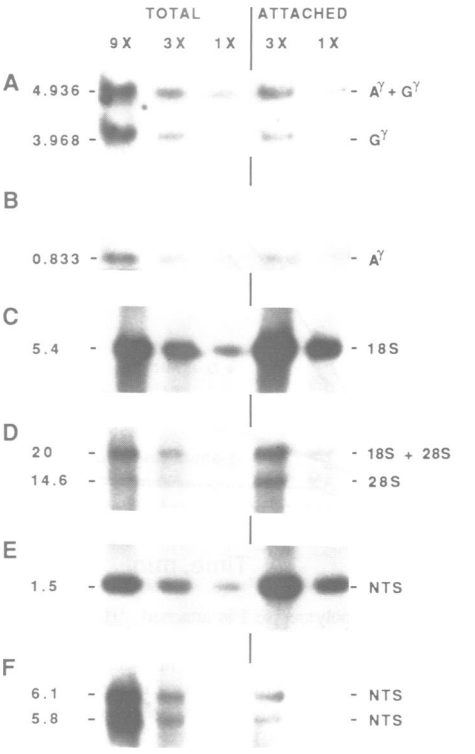

Fig. 3. Transcribed rDNA sequences are attached in HeLa cells. Labelled HeLa cells were encapsulated (5×10^6/ml), lysed and washed. Half the sample was digested with *Pst*I (500 units/ml), half without and both halves were incubated (32°C; 1 h). Subsequent electrophoresis in the isotonic buffer removed no label (i.e. none of the chromatin) from the undigested sample, but 82% from the digested sample. DNA was purified from both samples (to give 'total' and 18% 'attached' DNA) and redigested with *Pst*I (4500 units/ml; 16 h). Known amounts of DNA (i.e. 9×, 3× and 1×) were subjected to electrophoresis (in **A** and **B**, 1× DNA is 1 μg; in **C**−**F** 1× is 0.5 μg), blotted and hybridized with various probes. Photographs of the relevant parts of the resulting autoradiographs are illustrated. Fragment sizes (kb) are shown on the left, and their origin (i.e. globin or rDNA; NTS, non-transcribed spacer) on the right. The filter used in C was stripped and reprobed in F. (**A,B**) Globin locus (probe 3.2 kb *Hin*dIII fragment). Comparison of equal loadings (i.e. 1× with 1×) shows that the intensity of the attached samples is <1.1× that of total DNA (i.e. globin genes are not closely associated with the skeleton). (**C,D**) Transcribed part of the rDNA locus (probe: 5.7 kb *Eco*RI fragment from 18S human rDNA, which cross-hybridizes with 20 and 14.6 kb *Pst*I fragments). The intensities of all three bands given by 1× attached DNA is more than that of 3× total DNA (i.e. these transcribed genes are closely associated with the skeleton). (**E**) NTS from rDNA (probe: *Hae*III fragment). The 1.5 kb band is enriched in the attached sample. (**F**) NTS from rDNA (probe: 11.7 kb *Eco*RI fragment). The two fragments are only slightly enriched in the attached sample. Enrichments for the various bands were (top to bottom): 1.1× and 0.8× (A), 0.7× (B), 4.6× (C), 2.8× and 3.3× (D), 5.9× (E) and 1.5× and 1.7× (F) respectively.

RNA polymerase I resists electroelution

We next determined whether RNA polymerase I activity was retained within beads after removing most chromatin. We have shown previously that little, if any, of the total activity initially found in lysed cells is lost during washing or electroelution (Jackson and Cook, 1985b). The bulk of this activity is due to RNA polymerase II, with polymerase I accounting for about 15% of the total. These fractions are distinguishable using suitable concentrations of inhibitors. Polymerase I is very resistant to α-amanitin and very sensitive to actinomycin D; polymerases II and III are more sensitive to α-amanitin but more resistant to actinomycin D (Sollner-Webb and Tower, 1986).

The various polymerases are assayed by incorporation of [^{32}P]UMP into acid-insoluble material. Since there is no initiation under these conditions, the endogenous polymerases elongate pre-existing transcripts at a rate dependent on the UTP concentration; here we deliberately use low concentrations to prevent the synthesis of very long transcripts that might be made from sequences not transcribed *in vivo* (see Materials and methods). (The experiments now discussed are generally described as 'run-on' experiments using inefficient conditions; we prefer not to use this terminology as it implies that the polymerase is mobile).

Figure 4 illustrates a typical experiment. As has been shown previously (Jackson and Cook, 1985b), removal of all but 25% of the chromatin had little effect on the total polymerizing activity (cf. curves 1 and 2). (The slight drop is probably due to the truncation of the template). High concentrations of α-amanitin inhibited polymerases II and

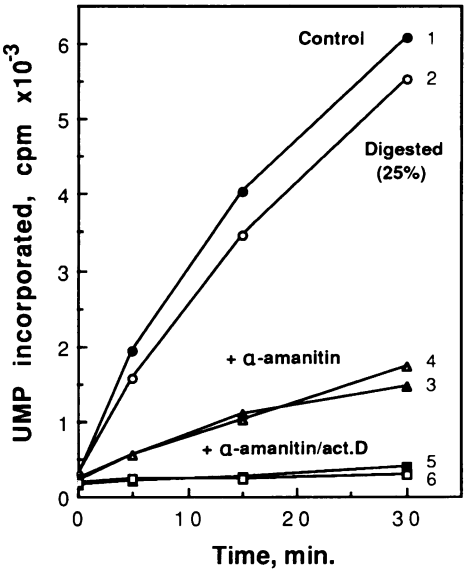

Fig. 4. Active RNA polymerase I is attached. ^{3}H-labelled cells were encapsulated (5×10^{6}/ml), lysed and washed, incubated with or without *Eco*RI (2500 units/ml; 32°C; 30 min) and then detached fragments electroeluted. The time-course of incorporation of [^{32}P] UTP (25μCi/ml; 'medium efficiency' conditions) into acid-insoluble material by beads treated in various ways is shown. In some cases cells were treated with actinomycin D (0.08 μg/ml; 30 min) before harvesting, in others lysed cells were preincubated with α-amanitin (200 μg/ml; 15 min; 4°C) before transcription. **Curve 1**: control, without inhibitors, digestion with *Eco*RI or electrophoresis. **Curve 2**: without inhibitors, but digested and electroeluted (25% chromatin remained). **Curve 3**: without digestion or electrophoresis, but with α-amanitin. **Curve 4**: with digestion, electroelution (25% chromatin remained) and α-amanitin. **Curve 5**: without digestion or electrophoresis, but with actinomycin D and α-amanitin. **Curve 6**: with digestion, electrophoresis (25% chromatin remained), actinomycin D and α-amanitin.

Table I. Initial rates of RNA synthesis by lysed cells after digestion with or without *Eco*RI and electrophoresis

Sample	Electrophoresis (% remaining)		α-amanitin (200 μg/ml)	Pretreatment: Actinomycin D −	Actinomycin D +	RNase −	RNase +
Undigested	+	(100%)	−	0.74	0.66		
Digested	+	(25%)	−	0.58	0.49		
Undigested	−	(100%)	+	0.08	0.009		
Digested	−	(100%)	+	0.10	0.007		
Undigested	+	(100%)	+	0.13	0.017		
Digested	+	(25%)	+	0.14	0.017		
Undigested	+	(100%)	−			0.45	0.41
Digested	+	(23%)	−			0.38	0.27
Undigested	+	(100%)	+			0.16	0.15
Digested	+	(23%)	+			0.11	0.12

Initial rates (pmol UMP incorporated/10^{6} cells/min) were determined over the first 5 min under conditions of 'medium efficiency', immediately after preincubation with or without α-amanitin (200 μg/ml; 4°C; 15 min). Two typical experiments are shown. In the first, some cells were grown in actinomycin D (0.08 μg/ml; 30 min) immediately prior to harvesting. In the second, some lysed cells were incubated with RNase (5 μg/ml; 15 min) prior to electrophoresis to remove >95% nascent RNA (labelled in 10 min *in vivo* by [^{3}H] uridine; Jackson and Cook, 1985b). Polymerase I activity is the activity remaining after preincubation with α-amanitin; it is sensitive to actinomycin D.

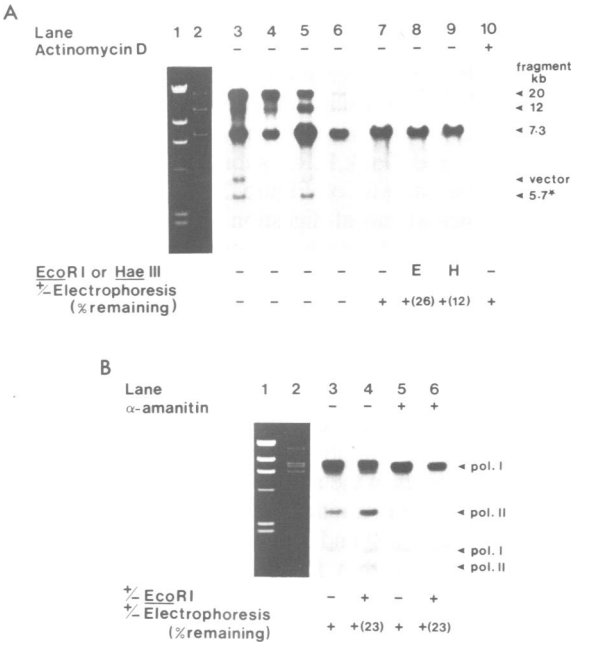

Fig. 5. Transcription is (A) authentic and (B) not due to polymerase II. ^{3}H-labelled cells, untransfected (**A**) or transfected with pSVα1Fd (**B**) were encapsulated (2.5×10^{6}/ml; except for A, lanes 4−7, at 10^{7}/ml), lysed, incubated (30 min; 32°C) with or without *Eco*RI (2500 units/ml) or *Hae*III (500 units/ml), detached fragments electroeluted and the percentage of ^{3}H (i.e. chromatin) remaining determined. Beads were recovered and [^{32}P]RNA made (30 min) under 'low efficiency' conditions (except for A, lanes 3 and 4 where 'medium efficiency' conditions were used). The concentration of specific [^{32}P] RNA made *in vitro* was assayed by hybridization with rDNA fragments on filters; after washing, autoradiographs were prepared (exposures 2 weeks, except for A, lanes 4 and 6 which were 2 days) and photographed. (**A**) Hybridization of [^{32}P]RNA made *in vitro* (from normal HeLa derivatives) with cosmid DNA fragments containing the rDNA locus (i.e. cos 7/*Eco*RI; fragment sizes on the right; *, most of the transcribed leader and 18S sequences were lost from this cosmid during cloning). Lanes 1 and 2: photographs of ethidium-stained gels containing λ/*Hin*dIII markers and rDNA plus vector fragments. Lanes 3−6: RNA made *in vitro* under conditions of 'medium efficiency' (lanes 3,4) hybridizes with rDNA sequences throughout the locus; cross-hybridization with vector sequences is evident on some longer exposures (this is not a general problem as all filters contained higher concentrations of λ/*Hin*dIII DNA that showed no cross-hybridization). RNA made *in vitro* under 'low efficiency' conditions (lanes 5,6), better reflects the pattern *in vivo*. Lanes 7−9: cutting plus electroelution removes chromatin with little effect on [^{32}P] rRNA concentration. Lane 10: as lane 7, but rRNA synthesis *in vivo* was inhibited by growing cells in actinomycin D (0.08 μg/ml) for 30 min before harvesting. The intensities given by the 7.3 kb bands in lanes 8−10 relative to that in lane 7 were 93%, 71% and <5% respectively (determined by quantitative densitometry of a shorter exposure). (**B**) Hybridization of [^{32}P] transcripts made *in vitro* (from transfected Cos cell derivatives) with cosmid DNA fragments containing the rDNA locus (i.e. cos 7/*Bam*HI; the transcription units carried by the fragments are indicated on the right). Lanes 1 and 2: photographs of ethidium-stained gels with λ/*Hin*dIII markers and cosmid fragments. Lanes 3,4: cutting plus electroelution removes chromatin with little effect on the concentration of transcripts hybridizing to polymerase I or II transcription units. Lanes 5,6: as lanes 3,4 except that transcription *in vitro* was carried out after preincubating (30 min; 4°C) lysed cells with α-amanitin (20 μg/ml) to inhibit transcription by polymerase II.

III. The α-amanitin-resistant activity (i.e. polymerase I activity) also resisted electroelution (curves 3 and 4). This resistant activity was eliminated by treating cells with a very

low concentration of actinomycin D (curves 5 and 6) confirming that it was due to rRNA synthesis. Polymerase II accounts for ~85% of the initial rate of synthesis and polymerase I for ~15% (Table I). Bulky transcripts—or perhaps more importantly, nascent RNA−protein interactions involved in nucleolar architecture—cannot prevent polymerase I from electroeluting, as pre-treatment with sufficient RNase to remove >95% nascent RNA has little effect on its retention (Table I).

The authenticity of these transcripts was monitored as follows (Figure 5). RNA was made *in vitro* using [^{32}P] UTP. This RNA, containing transcripts from all parts of the genome, was then hybridized with filter-bound rDNA fragments which span most of the ribosomal locus; after autoradiography, the intensity of hybridization with rDNA then reflects the concentration of nascent rRNA in the mixture. When 'medium efficiency' conditions were used during transcription, the [^{32}P]RNA hybridized not only with genic regions but also with the 'nontranscribed spacer' (i.e. the 20 and 12 kb fragments, lanes 3 and 4); as transcription should terminate about 1 kb into the larger fragment the intensity of the 20 kb signal suggests that transcription has not terminated properly *in vitro*. (In conventional terminology, the polymerase has 'run-on' into the nontranscribed spacer). This effect is minimized by using 'low efficiency' conditions where the elongation rate ('run-on') is reduced (lanes 5−10). Then, few transcripts hybridized with 'nontranscribed spacer' fragments; most hybridized with the genic region contained in the 7.3 kb fragment (lane 6). Therefore these conditions were used to see if removing chromatin affected the concentration of rRNA made *in vitro*.

Removal of most chromatin, by cutting with *Eco*RI or *Hae*III and electroelution, had little effect on the intensity of hybridization to the genic region (Figure 5A, compare lane 7 with 8 or 9). The observed decreases, to 93 and 71% in lanes 8 and 9 respectively, may be due to template truncation: there are numerous *Hae*III sites in the GC-rich ribosomal locus. This hybridization was sensitive to a low concentration of actinomycin D (lane 10). These results confirm that little active polymerase is lost and that this activity is due to polymerase I.

Figure 5B confirms that we are dealing with authentic polymerase I transcripts. Cos cells were transfected with a plasmid bearing two polymerase II transcription units (i.e. the α-globin gene and the SV40 early promoter attached to pBR322 sequences). 2.5 days later, when these units were active, cells were lysed and [^{32}P]RNA made *in vitro* as before. This RNA now contains labelled pBR322 sequences initiated at the SV40 promoter by polymerase II, in addition to monkey rRNA initiated by polymerase I. This can be demonstrated by hybridizing it with fragments from the cosmid used previously (cut with a different enzyme). Removing chromatin, as before, had little effect on the intensity of hybridization with any of the fragments, confirming that both polymerase I and II resisted electroelution (Figure 5B, lanes 3,4). Polymerase II transcripts were reduced by pretreatment with α-amanitin while the polymerase I transcripts were unaffected (lanes 5,6).

These experiments show that active polymerase I behaves like polymerase II in resisting electroelution.

Discussion

Organization of rDNA

Nucleolar activity is closely linked with cell growth; the number of fibrillar centres increases in proportion to growth rate in human fibroblasts (Jordan and McGovern, 1981) and highly active lymphoblasts have similar numbers of fibrillar centres as active rRNA genes (Ochs and Smetana; 1989). Conversely, micro-injection of antibodies directed against RNA polymerase I eliminates pre-nucleolar bodies and hence nucleoli (Benavente *et al.*, 1987). At a more molecular level, active ribosomal chromatin is sensitive to nucleases (Davis *et al.*, 1983; Ness *et al.*, 1988; Conconi *et al.*, 1988). These structural differences probably permit rDNA fractionation on the basis of its differential solubility following nuclease digestion; the proportion of nucleosome-free, insoluble genes which pellet with residual nuclei correlates with nucleolar activity (Davis *et al.*, 1983). All these results—and others indicating that nascent RNA is associated with a dense fibrillar component (Fakan and Puvion, 1980; Wachtler *et al.*, 1989) whilst RNA polymerase I (and topoisomerase I) are associated with fibrillar centres (Scheer and Rose, 1984; Reimer *et al.*, 1987)—imply that rDNA activity depends on a precise organization relative to large sub-nucleolar structures.

The association of rDNA with different chromatin structures is also evident here (Figure 2). About 1/3 of the ribosomal locus is particularly sensitive to digestion; the 7.3 kb rDNA fragment is released at much lower enzyme concentrations than those required to release the inactive β-globin fragments; the latter are contained in fragments with a stable canonical nucleosomal repeat (cf. Figure 2B, lanes h and p). No partial digestion products are obtained from the accessible fraction of ribosomal repeats, implying that all sites within it are equally accessible. Paradoxically, those ribosomal loci that resist digestion have sites which are completely inaccessible, even more so than those in inactive globin genes. Therefore the experiment described in Figure 2 reveals three different chromatin structures: an accessible rDNA fraction (presumably the active fraction), the relatively inaccessible globin gene and a completely inaccessible rDNA fraction.

Active rDNA and RNA polymerase I are attached

A variety of studies have shown that transcriptionally active ribosomal genes are associated with a subnuclear fraction that pellets, whether isolated using hypotonic (Davis *et al.*, 1983), hypertonic (Jackson *et al.*, 1984b) or sequential hypo- and hypertonic treatments (Pardoll and Vogelstein, 1980; Keppel, 1986). These results were criticized because ribosomal chromatin, rich in nascent RNA, might artifactually aggregate under such unphysiological conditions. We sidestep this problem using conditions more similar to those found *in vivo* (Jackson *et al.*, 1988). When incubated under appropriate conditions, our preparation replicates and transcribes at rates close to those found *in vivo* and retains a stable domain organization with little, if any, nucleosomal rearrangement (Jackson *et al.*, 1990). We have confirmed that a fraction of ribosomal repeats (the active fraction) remains associated with residual nuclei when most chromatin is removed (Figure 3). Moreover, >90% of active RNA

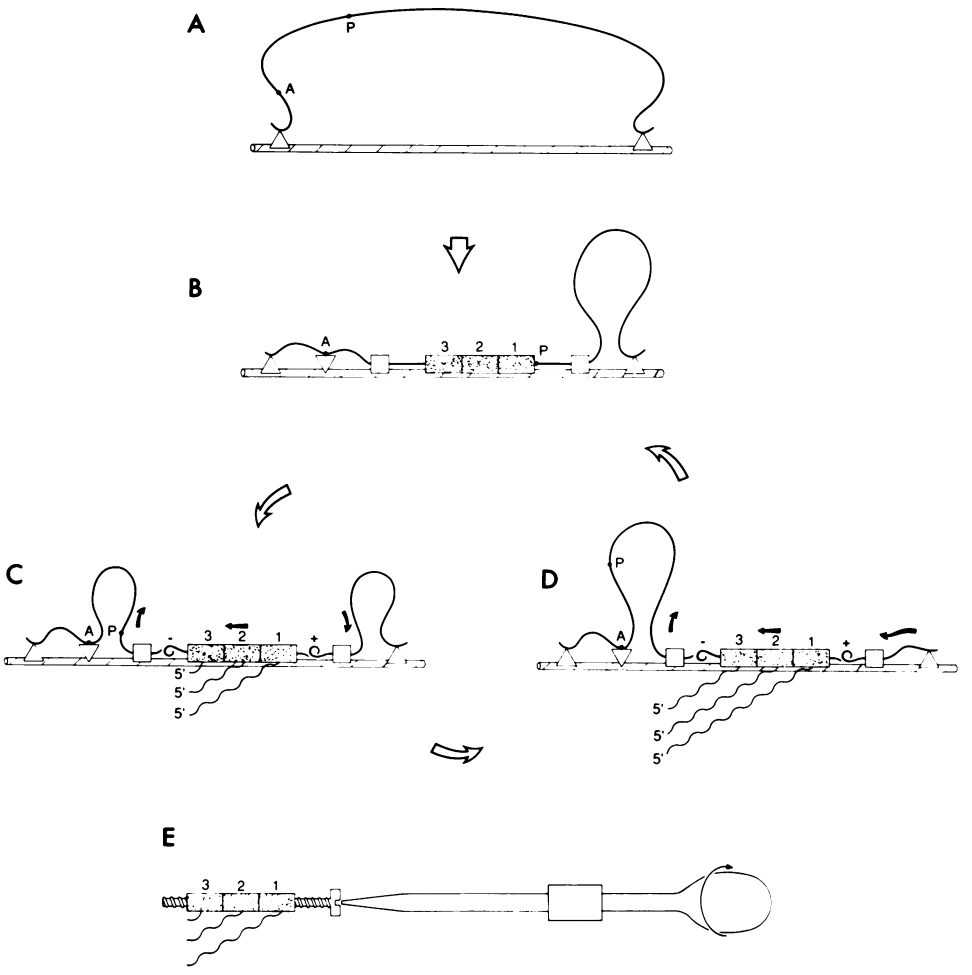

Fig. 6. A schematic model for transcription (not to scale). (**A**) A loop of rDNA is shown attached to the skeleton (rod) at two sites (△), which probably persist whether or not the loop is transcribed. rDNA cannot be transcribed as it is remote from any attached polymerase. (A) marks an upstream activating sequence and (P) the promoter. (**B**) On activation, the loop binds to the skeleton and assembles into an attached transcription complex containing, in this example, 3 closely-spaced polymerases (stippled rectangles, 1−3), an upstream binding site (▽) and topoisomerases (squares). For the sake of simplicity, these proteins are drawn spatially separated, but probably abut each other; attached transcription factors, further sites like A and RNA processing enzymes are also omitted. The upstream binding site now permanently tethers A to the complex. (**C,D**) After initiation, DNA moves (filled arrows) through the complex and RNA (wavy line) is synthesized; as a result the loop on the right shrinks and that on the left grows. The 5′ RNA end is also probably attached, rather than as shown. Positive and negative supercoils would appear as shown if not removed by the two topoisomerases; if these abut the polymerases, no supercoils would appear. After the transcript is completed, A remains attached so the DNA can easily return to its position in (B) and re-initiate synthesis. (**E**) The rotational and lateral movements of DNA in (C) are analogous to a bolt (DNA) being driven through 3 fixed nuts (polymerases) by a ratchet screwdriver. Wavy lines below the nuts mark the analogous positions of transcripts. Each of the 3 nuts 'sees' the thread as it passes through; the fixed polymerases 'see' the transcribed strand in the same way. As a right-handed twist drives the bolt (arrow), a right-handed twist accompanies DNA translocation and just as spinning the ratchet relieves wrist-strain, so a topoisomerase spins the DNA to release superhelical tension. Another ratchet (not shown) at the left releases tension of opposite sense. As the bolt also rotates at each nut at the same speed, no torsional tension builds up between nuts. Similarly, no supercoils usually accumulate anywhere in the transcription unit.

polymerase I is also retained, even when essentially all nascent RNA is removed (Table I).

A model for transcription by RNA polymerase I

These facts concerning the structural organization of nucleoli and active polymerase I are consistent with the transcription machinery being attached in the fibrillar centres. Then, rDNA in the dense fibrillar component would associate with the transcription machinery in the fibrillar-centre; RNA would be synthesized by an attached polymerase and the resulting rRNA would pass out towards the dense fibrillar and granular components as it matures.

In contrast to the accepted model for transcription where RNA is made as a mobile polymerase processes along the DNA, the polymerase here is immobile and RNA is synthesized as the DNA moves past it (Figure 6; see also Cook, 1989). Then DNA must rotate as it moves laterally so that the transcribed base on the helical template strand maintains the same topological relationship to each fixed polymerization site in an array. A transcribed repeat can be imagined as being connected to adjacent sequences through two ball-bearing races—topoisomerases—that allow it to rotate. Once the DNA has passed through the array it disengages from the polymerizing sites whilst remaining locally attached (i.e. at A in Figure 6); then it re-engages with the first site in the array to start the process again.

This model is consistent with the appearance of 'Miller' spreads, if it is assumed that the skeleton is destroyed or

detached from transcription complexes during spreading. In spreads, polymerases are either tightly packed (e.g. spaced about 100 bp apart in HeLa), or absent (Miller and Beatty, 1969; McKnight and Miller, 1976; Puvion-Dutilleul *et al.*, 1978; see also Davis *et al.*, 1983) so synthesis must be regulated at initiation rather than elongation (Cox, 1976; Grummt *et al.*, 1976; Sollner-Webb and Tower, 1986) as in Figure 6. The model offers new explanations of why transcription complexes are so stable (Henderson and Sollner-Webb, 1986; Grummt *et al.*, 1976; McStay and Reeder, 1986; Sollner-Webb and Tower, 1986), why topoisomerase I cuts are concentrated at the ends of each repeat (Gocke *et al.*, 1983; Fleischmann *et al.*, 1984; Gilmour and Elgin, 1987; Ness *et al.*, 1988), for phenomena such as transcriptional 'interference' and polymerase 'handover' (Henderson *et al.*, 1989; Lucchini and Reeder, 1989) as well as suggesting how enhancers might work as attachment sequences (Cook, 1989).

This model begs the question; to what are the transcription complexes attached? It may be that the nucleus, like the cytoplasm, contains a number of different skeletons. Using conditions identical to those used here (i.e. nuclease digestion and electroelution), one nucleoskeleton has been visualized. This supports residual clumps of chromatin and connects the nucleolus to the nuclear periphery; it has the morphology of intermediate filaments (Jackson and Cook, 1988). Unfortunately nucleoli were so dense that it was impossible to see if intermediate filaments penetrated into them, but if active RNA polymerase I is attached to such filaments the generation of 'Miller' spreads may be explained; some of these filaments dissolve in the hypotonic conditions used for spreading (Zackroff and Goldman, 1979).

Materials and methods

Cell encapsulation and lysis

Conditions for cell growth, encapsulation and lysis have been described (Jackson *et al.*, 1988; Jackson,D.A., Dickinson,P. and Cook,P.R., submitted). In most experiments cells were grown for 18–24 h in [methyl-^{3}H] thymidine (0.05–0.25 μCi/ml; ~60 Ci/mmol) to label uniformly their DNA. This enabled (i) corrections to be made subsequently for any slight variations in cell numbers (Jackson and Cook, 1986), (ii) determination of the percentage of chromatin resisting electroelution and (iii) equal amounts of total and digested DNA to be applied to gels. Radioactivity in encapsulated cells was determined after incubation with RNase A (10 μg/ml; 30 min; 37°C), then 0.5% SDS plus proteinase K (500 μg/ml; 3 h; 55°C) and 1M HCl (10 min; 95°C). Encapsulated cells were lysed (3 × 20 min at 4°C) using Triton X-100 in the 'physiological' buffer at pH 7.4 (containing 22 mM Na^+, 130 mM K^+, 1 mM Mg^{2+}, <0.3 μM free Ca^{2+}, 132 mM Cl^-, 11 mM phosphate, 1 mM ATP and 1 mM dithiothreitol) and washed (5 × 10 min; 4°C) in the buffer.

Digestion with nucleases and electroelution

Labelled cells were encapsulated, lysed, washed and incubated in an equal volume of buffer with various restriction enzymes. Chromatin was electroeluted (generally 16 h at 1–2 v/cm, but 5 h at 3 v/cm when transcription assays followed) from beads by applying the beads to wells in a 0.8% agarose gel in the 'physiological' buffer. Subsequently the beads were recovered.

Probes

(i) The 3.2 kb *Hin*dIII fragment from the human A^γ gene cloned in pBR322; this hybridizes with three major *Pst*I bands, one by cross-hybridization to G^γ, and two *Eco*RI bands. Other probes were isolated from a cosmid (cos r5) which contains most of the human rDNA locus (isolated in this laboratory; unpublished results). (ii) A gel-purified 7.3 kb *Eco*RI fragment containing part of the 28S gene. (iii) A gel-purified 5.7 kb *Eco*RI fragment containing part of the 18S gene; it also hybridizes with the 14.6 kb *Pst*I fragment containing the 28S gene. (iv) A gel-purified *Hae*III fragment cut from the 11.7 kb *Eco*RI fragment from the non-transcribed spacer (NTS); this detects the 1.5 kb *Pst*I NTS band. (v) An 11.7 kb *Eco*RI fragment from the NTS which hybridizes strongly to two fragments.

Transcription assays

General methods for assays are described by Jackson and Cook (1985b). Beads were resuspended in an equal volume of 'physiological' buffer and transcription assays (500 μl; 32°C) conducted in the buffer supplemented with 250 μM ATP, CTP and GTP plus 0, 1, or 125 μM UTP, 50 μM S-adenosyl methionine, 2.5 mM $MgCl_2$ and 50 μCi/ml [^{32}P]UTP (~3000 Ci/mmol). [^{32}P]RNA is synthesized using 1 μM UTP at about 100 nucleotides/min ('medium efficiency' conditions) or ~5% of the maximum obtainable with this system using 125 μM UTP (Jackson *et al.*, 1988). With no unlabelled UTP, the rate is ~2 nucleotides/min ('low efficiency' conditions). The relative proportions of actinomycin- and α-amanitin-sensitive transcription (i.e. due to polymerases I and II) remains roughly constant, irrespective of triphosphate levels (results not show). As the reciprocals of transcription rates and triphosphate concentrations are linearly related (results not shown), the same numbers of enzyme molecules (i.e. the majority of those active *in vivo*) must be active *in vitro* even under 'moderate' and 'low efficiency' conditions.

In Figure 5B, Cos cells were transfected with plasmid pSVα1Fd (2 μg plasmid DNA plus 20 μg HeLa DNA cut with *Hae*III) as described by Briggs *et al.* (1989). This plasmid replicates so that after 2.5 days, cells contain an average of 100–200 copies, with 5–10% of the cells containing plasmid. The two polymerase II promoters (i.e. globin and SV40 early) in the plasmid are active, with most transcripts being derived from the SV40 promoter and downstream vector (pBR322) sequences (Briggs *et al.*, 1989). Therefore, polymerase II transcripts—initiated *in vivo* on transfected pSVα1Fd and labelled by elongation *in vitro*—hybridize with vector (pBR322) sequences in the rDNA cosmid.

Hybridization

DNA was purified from encapsulated and lysed cells using a Gene Clean kit (Bio 101 Inc., La Jolla) according to the manufacturer's instructions, which involves dissolving the DNA in saturated NaI, phenol extraction and recovery on 'Glassmilk'. DNA fragments were sized by gel electrophoresis; after electrophoresis the gel was stained with ethidium, photographed, blotted and hybridized with the appropriate probes (Cook and Brazell, 1980).

[^{32}P]RNA synthesized in a transcription assay was purified by washing beads in 10 mM Tris (pH 8.0), 1 mM EDTA, 50 mM NaCl to remove unincorporated label before treatment with RNase-free DNase (20 μg/ml; 15 min; 32°C). tRNA was added to 20 μg/ml, and beads incubated in 1% SDS with proteinase K(50 μg/ml; 16 h; 37°C). After spinning to remove agarose (25 000 r.p.m.; 20 min), the supernatant was extracted with phenol/chloroform and precipitated with ethanol. >95% of the incorporated label was recovered. After redissolving and dialysis against 10 mM Tris (pH 8.0), 1 mM EDTA, retreatment with DNase and reprecipitation, 60–80% of the incorporated label and <10% bulk DNA was recovered. [^{32}P] RNA ($0.5–2 \times 10^6$ c.p.m.) was dissolved in 1 ml hybridization buffer and hybridized (42 h; 42°C) to filter-bound cosmid (cos 7) DNA fragments (the cosmid contains most of the rDNA locus); filters (~8 × 4 cm) were washed and autoradiographs prepared (Jackson and Cook, 1986).

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