Manuscript no.: JCS8319

Journal of Cell Science 105, 1143-1150 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Transcriptionally active minichromosomes are attached transiently in nuclei through transcription units

D. A. Jackson and P. R. Cook*

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, UK

*Author for correspondence

SUMMARY

Viral minichromosomes provide simple models for chromatin domains. The sequences attaching them to larger nuclear structures were mapped; attachments were defined operationally by their ability to prevent chromatin fragments electroeluting from nuclei. Cells encapsulated in agarose microbeads were lysed and analysed subsequently in a 'physiological' buffer. Non-transcribed minichromosomes eluted from nuclei but transcriptionally active ones did not. Cutting attached minichromosomes with *Hae*III enabled most of the resulting ~400 bp fragments to elute. Analysis of residual fragments showed that no single sequence was responsible for attachment; rather, minichromosomes were attached at only one or two points through a promoter or part of a transcription unit. This suggests that RNA polymerases mediate attachments that change dynamically as the template slides past the attached enzyme. As, under optimal conditions, polymerases continue to elongate roughly at the rate found in vivo, these attachments are unlikely to be generated artifactually.

Key words: chromatin domains, chromatin loops, RNA polymerase II/transcription

INTRODUCTION

Views on how the metabolism of RNA within the nucleus is organized are changing, largely as a result of the introduction of high-resolution immunofluorescence. Traditional views were based on the assumption that the enzymes involved were soluble and freely diffusible: then sites of RNA synthesis and processing should be spread throughout nuclei. However, recent studies point to a structured pathway in which transcripts are synthesized and processed on an underlying solid phase (reviewed by Cook, 1989; Huang and Spector, 1991; Jackson, 1991; Carter et al., 1991). For example, transcription sites and processing components are not diffusely spread throughout euchromatin but focally concentrated in discrete 'speckles'; moreover, nascent transcripts appear to be exported to the nuclear periphery along tracks (e.g. see Lawrence et al., 1989; Carmo-Fonseca et al., 1991a,b; Huang and Spector, 1991; Carter et al., 1991; Jackson et al., 1993; Xing et al., 1993).

How the template - in the form of a chromatin loop - might be incorporated into this transcriptional pathway is controversial (Cook, 1988). The controversy stems partly from the innate complexity of nuclear structure. This is well illustrated by 'position' effects: insertion of reporter genes at different chromosomal sites in transgenic animals clearly shows that structure and activity depend on the precise chromosomal 'context' provided by flanking cellular chromatin (reviewed by Bonnerot et al., 1990). Additionally, chromatin is so highly concentrated within nuclei that novel (i.e. artifactual) attachments might be created when ionic conditions are altered.

We have analysed viral minichromosomes of ~5 kb because they provide simple models of chromatin domains free of the influence of flanking chromatin. pSVα1fd (Fig. 1) possesses the SV40 origin of replication and multiplies in cos7 cells expressing large T antigen. It carries two RNA polymerase II transcription units containing the SV40-early and human α1-globin promoters; transcription from the latter yields high levels of authentic globin mRNA independently of the linked SV40 enhancer (Mellon et al., 1981; Charnay et al., 1984). Transfected cells are encapsulated in agarose microbeads and then lysed with Triton in a 'physiological' buffer. Encapsulation allows lysed cells to be handled freely without aggregation; at the same time chromatin is accessible to proteins like restriction enzymes (Jackson et al., 1988).

We have analysed which sequences attach minichromosomes to host nuclear structures; attachments are defined operationally by their ability to prevent chromatin fragments from electroeluting from nuclei. We first established conditions yielding a relatively homogeneous population of transcriptionally active minichromosomes. Then, after cutting with nucleases, fragments resisting electroelution from beads were analysed, using the physiological buffer throughout. Most cellular chromatin elutes to leave residual clumps attached to an underlying skeleton (Jackson and Cook, 1988). The 'speckled' transcription sites also resist elution, presumably because they are also attached - directly

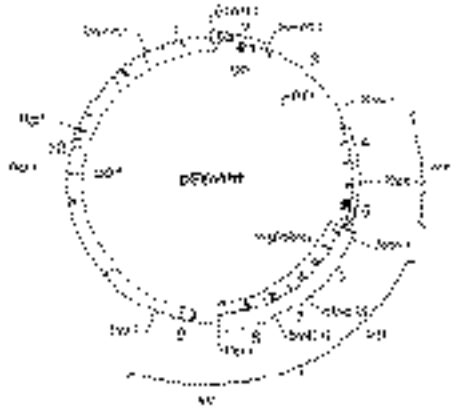

Fig. 1. pSVα1fd. The SV40 origin (ori) and α1-globin insert are indicated; hatched areas contain promoters and the widest regions are the transcribed parts of the natural transcription units. Transcription (arrow) from the globin promoter terminates at the natural poly(A) site but transcription (arrow) from the SV40 promoter reads through into pBR322 (pBR) sequences. Regions 1-10 were isolated using the restriction sites shown on the outside. a-f are single-stranded fragments from the regions shown (a,c,e and b,d,f are − and + strands, respectively); 47 *Hae*III sites and 4 additional *Bgl*I sites are indicated by short and long ticks on the inside of the circle.

or indirectly - to the skeleton (Jackson et al., 1993). Two populations of minichromosomes were found; an inert fraction that eluted (i.e. was unattached) and a transcribed fraction that resisted elution due to attachment at a promoter or an active polymerase. This suggests that active transcription complexes mediate attachments.

All approaches involving sub-cellular fractionation face the criticism that any attachments seen are generated artifactually. This criticism can never be completely answered by any biochemist who breaks open a cell. However, the use of physiological conditions throughout and recovery of essentially all the polymerizing activity found in vivo - even after cutting and elution (Jackson et al., 1988) - make explanations based on an artifactual aggregation of polymerases difficult to sustain; aggregation would be expected to reduce activity.

MATERIALS AND METHODS

Plasmids

pSVα1fd was constructed from pBR322 by: (i) deleting sequences (1420-2490) poisonous to replication in mammalian cells; (ii) inserting the 311 bp *Eco*RII G fragment containing the SV40 origin using *Eco*RI linkers (Myers and Tjian, 1980); and (iii) inserting the 1502 bp *Pst*I fragment containing the human α1-globin gene between the *Pst*I and *Pvu*II sites, with loss of the 5′ *Pst*I site. Regions 1-10 (Fig. 1) are 375, 320, 515, 416, 304, 389, 199, 305, 358 and 234 bp, respectively. Single-stranded M13 clones (Fig. 1) were provided by E. Whitelaw (Briggs et al., 1989). pMS5 contained the 7.3 kb human rDNA *Eco*RI fragment cloned into the *Eco*RI site of pBR322.

Cell culture and transfection

cos7 cells were grown in DMEM + 5% foetal and 5% newborn calf serum (Gibco-BRL) and transfected with 0.02-25 μg plasmid DNA (>80% supercoiled) plus 20 μg carrier HeLa DNA cut with *Hae*III, per 75 cm^2 flask, glycerol-shocked 6 h later, fresh medium added, passaged after 18 h and grown for 24 h (Mellon et al., 1981). This minimizes the background of precipitated DNA complexes and maintains sub-confluency. In most experiments transfected cells were grown for 18-24 h in [*methyl*-^{3}H]thymidine (0.05-0.25 μCi/ml; ~60 Ci/mmol) prior to harvesting, to label their DNA uniformly, to enable: (i) corrections to be made subsequently for any slight variations in cell numbers; (ii) determination of the percentage of chromatin resisting elution; and (iii) equal amounts of total and digested DNA to be applied to gels and filters (Dickinson et al., 1990). Transfection efficiencies were determined using a plasmid that expresses β-galactosidase, L27βGal (Sanes et al., 1986).

Cell encapsulation, lysis and chromatin fragmentation

Cells were encapsulated (5×10^6 to 20×10^6/ml) in 0.5% agarose microbeads, incubated in medium (1 h; 37˚C) and lysed in physiological buffer (Jackson et al., 1988) containing 0.5% Triton X-100 (15 min). Physiological buffer (pH 7.4) contains 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. Then beads were washed (5×; 10 vol.; 0˚C) in buffer without Triton over 15-30 min and digested with a restriction endonuclease (500-2,000 units/ml; 32˚C; 15 min), washed and soluble chromatin fragments electroeluted (4-5 h; Jackson and Cook, 1985a, 1988).

Transcription

Beads in physiological buffer were pre-incubated (32˚C, 5 min) and transcription was initiated by adding a 10× mixture to give final concentrations of 100 μM GTP, CTP, ATP and 100 μCi/ml [^{32}P]UTP (400-3000 Ci/mmol). At this limiting UTP concentration the elongation rate is 3 nucleotides/min. After elongation for 10 min, 100 μM UTP was added for 2 min to elongate nascent chains without further incorporation of ^{32}P. In some experiments, elongation rates and hence label inputs were increased ~5× by adding 0.1 M $(NH_4)_2SO_4$ (Jackson and Cook, 1985b); this had no effect on resulting hybridization patterns. Samples subjected to electrophoresis, whether treated with nucleases or not, were 25-50% less active than controls stored at 4˚C.

RNA was purified from beads by washing (5×, 10 ml) to remove unincorporated label; 1.5 ml TEN (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl) was added to 0.5 ml packed beads, NaOH was added (0.2 M; 10 min, 0˚C) to hydrolyse RNA into small pieces, 1 M HCl was added to neutralize, followed by 2 ml TEN + 0.5% SDS. After incubation (1 h, 20˚C; this allows any plasmid circles and small fragments to diffuse out of beads), beads were pelleted (5000 ***g***; 20 min, 20˚C) with >95% genomic DNA (if intact). Carrier yeast tRNA (10 μg/ml) was added to the supernatant, RNA precipitated, redissolved and dialysed against TEN. After incubation with DNase (RNase-free; 50 μg/ml; 30 min; 37˚C), extraction with phenol/chloroform and reprecipitation, 60-90% ^{32}P is recovered as chains of 75-150 bp with <1% DNA.

Gel electrophoresis and hybridization

DNA and RNA preparation, gel electrophoresis, fragment recovery, preparation of [^{32}P]DNA probes (1 to 2.5×10^9 c.p.m./μg) by hexanucleotide priming, blotting and hybridization involved routine procedures (Sambrook et al., 1989). Filter hybridizations (24-36 h; 42˚C) involved 2×10^6 to 10×10^6 c.p.m. [^{32}P]DNA or 2×10^6 to 5×10^6 c.p.m. RNA labelled in vitro at 10^5 c.p.m./cm^2 filter and washing (3× 20 min in 2× SSC, 0.2% SDS at 20˚C then 2× 20 min in 0.2× SSC, 0.2% SDS at 67˚C). ‘Slot’ blots involved 2.2 to 200×10^3 cell equivalents of pure DNA/slot.

Table 1. Copy numbers of pSVα1fd in transfected cos7 cells

Input (μg)	% Cells transfected	Copies/ transfected cell	Relative globin message concentration	% Plasmid retained	Retained number of copies
0.2	2.5 ± 0.8	1464	1	78 ± 5	1141
1.0	5.7 ± 1.9	1842	3.8	69 ± 5	1271
5.0	13.9 ± 2.5	3043	12	39 ± 8	1186
25.0	21.3 ± 6.3	6338	21	31 ± 6	1965

Cells were transfected with different plasmid DNA inputs and harvested 2 days later. The percentage of transfected cells was determined using a plasmid expressing β-galactosidase. Copy numbers were determined (using autoradiographs like those in Fig. 2) by comparing signal intensities given by DNA from transfected cells with known numbers of plasmid DNA molecules run in the presence of 10^6 cell-DNA equivalents. Both sets of DNA were linearized with *Bam*HI. Relative globin message concentrations were measured by purifying total RNA, electrophoresis on a denaturing gel, blotting, probing with [^{32}P]region 6, autoradiography and comparative densitometry of the 9 S globin band. The increase seen with increasing input reflects the accumulation of completed transcripts rather than an increase in the number of active genes in each cell. The percentage of plasmids resisting electroelution was determined as in Fig. 2 and the retained number of copies calculated.

RESULTS

Growth and expression of pSVα1fd in cos7 cells

Between 12 and 60 h after $CaPO_4$-mediated transfection, pSVα1fd DNA doubles 5-6 times to give thousands of copies per cell (Table 1; Mellon et al., 1981). >97% of the progeny have been replicated in vivo, so any contribution from unreplicated input goes undetected. Although many are transcribed into authentic globin message (Table 1; Mellon et al., 1981), high inputs led to an accumulation of transcriptionally inert minichromosomes (see below), aberrant transcription and cell death (not shown). Consequently, low inputs (i.e. 0.2-1 μg DNA/plate) were generally used as a compromise between maximizing copy number and minimizing the number of inactive chromosomes.

pSVα1fd resists electroelution but is not entrapped in cellular chromatin

Very long chromatin fragments containing >100 kb DNA can electroelute from encapsulated and permeabilized cells (Jackson and Cook, 1985a), so 5 kb minichromosomes should elute easily, if they are unattached. At high inputs (and so high copy numbers) most eluted; in contrast, at low copy numbers few eluted (Table 1). Roughly the same number/cell were retained irrespective of initial input. Quite different results were obtained when functionally inert DNA was transfected (not shown). For example, with pBR322, ~50% of recovered DNA was degraded and >98% eluted; with a 4.5 kb *Pst*I β-globin fragment inserted into pMB9 (i.e. a plasmid with the globin promoter but no enhancer), >95% eluted.

Minichromosomes might resist elution simply because they were trapped in dense cellular chromatin. This was removed by cutting with *Bgl*II, which does not cut the minichromosomes, to see if they now eluted with cellular fragments. Retained plasmid sequences were identified after purifying DNA, electrophoresis, blotting and probing with a full-length plasmid probe. In uncut controls, autoradiography showed that >80% plasmid DNA was supercoiled and so derived from intact nucleosome-covered circles; <5% were slowly migrating replicative intermediates (e.g. Fig. 2, lanes 1,2). 18% of minichromosomes eluted from these uncut controls (Fig. 2 legend, lane 8;). After cutting with *Bgl*II, 72% cellular chromatin was lost but only 15% of (uncut) minichromosomes (Fig. 2, lanes 3,9). (Percentage losses of cellular chromatin were determined from losses of ^{3}H from cells pre-labelled with [^{3}H]thymidine.)

These experiments show that after transfection with 1 μg DNA cells contain ~1800 plasmids covered with nucleosomes. About ~1300 resist elution and so these are defined operationally as ‘attached’.

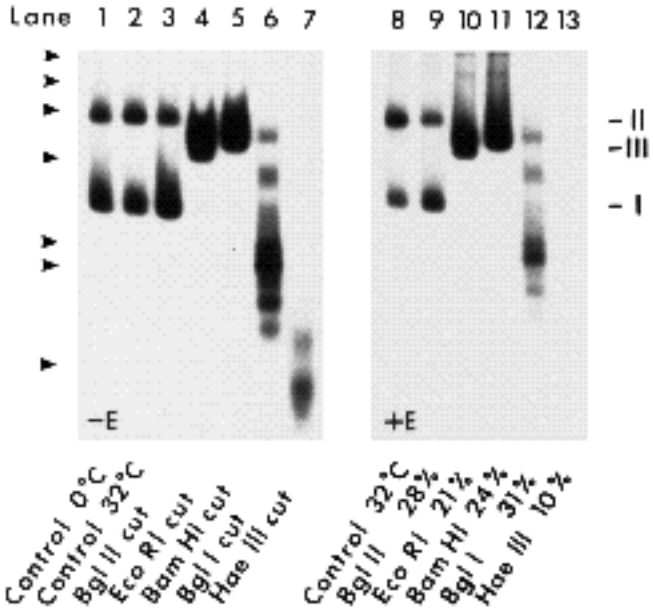

Fig. 2. Chromatin accessibility and attachment. Transfected cells were labelled with [^{3}H]thymidine, harvested after 42 h, encapsulated (2.5×10^6 cells/ml), lysed and incubated (1.25×10^6 cells/ml; 30 min) without (control, 0 or 32˚C) or with endonucleases (500-2000 units/ml; 32˚C). Some beads were subjected to electrophoresis (± E); DNA was prepared from equal quantities of beads, separated by electrophoresis, blotted, the resulting filters probed with [^{32}P]pSVα1fd DNA and an autoradiograph prepared (24 h exposure). The % chromatin remaining in beads was determined by scintillation counting (shown under right panel); 82, 85, 76, 80, 32 and 8% of plasmid DNA remained in samples 8-13, respectively (estimated by quantitative densitometry relative to lane 2). Positions of forms I, II, III and lambda/*Hin*dIII markers are indicated (arrowheads); φX174/*Hae*III markers were also used (not shown).

Table 2. The number of attachments per minichromosome

Exp.	Restriction enzyme	% Remaining attached	Number average size (kb)	Loop size (kb)	Attachments per molecule
1	*Hae*III	13	0.44	3.4	1.5
2	*Hae*III	12	0.51	4.1	1.2
3	*Hae*III	9.2	0.36	3.9	1.3
4	*Bgl*I	35	1.8	5.1	0.97
5	*Bgl*I	51	2.3	4.5	1.1
6	*Bgl*I	44	1.9	4.3	1.2

Several experiments like that described in the legend to Fig. 2 were analysed as described by Jackson et al. (1990a). The % of *Hae*III or *Bgl*I fragments that remained attached was determined by slot-blotting (Fig. 3). Weight-average fragment sizes were obtained by quantitative densitometry of autoradiographs (e.g. Fig. 2, tracks 12, 13) and no. average sizes derived. Loop sizes (i.e. distance between attachments) and number of attachments/molecule were then calculated. Thus, in experiment 1, *Hae*III fragments resisting elution had a no. average size of 0.44 kb and constituted 13% of intact plasmid DNA that resisted elution. Then, the average distance between attachment points is 100/13 (× 0.44) = 3.4 kb. As pSVα1fd is 5 kb long, there are 1.5 attachments/minichromosome. Values in experiments 4-6 are probably underestimated as the approach requires that at least one cut is made between each attachment point and *Bgl*I has only 6 sites. A preferential loss of smaller fragments during blotting would also give fewer attachments.

There are few attachments per minichromosome

The nuclease accessibility of different sites within minichromosomes was determined next (Fig. 2). *Bam*HI, which has only one site (Fig. 1), linearized >98% minichromosomes (lane 5) and *Eco*RI (2 sites) cut 95% into two (lane 4; the small *ori* fragment is visible after longer exposure). These sites are completely accessible, as expected of nucleosome-free sites close to the origin. In contrast, cutting with *Bgl*I (6 sites) or *Hae*III (47 sites) generated the partial-digestion products expected of nucleosome-covered DNA (lanes 6,7); for example, *Hae*III fragments were three times longer than those obtained by complete digestion of naked DNA (Table 2), so two out of three sites were inaccessible. As these sites remained inaccessible during prolonged digestion (not shown; see also Jackson et al., 1990a) nucleosome position must be stable in our buffer.

The proportion of attached fragments was determined by eluting unattached ones (Fig. 2, lanes 8-13). After *Bam*HI cutting, few linear minichromosomes were lost, despite removal of 86% cellular chromatin (cf. lanes 8,11; and legend). Most were not attached at the origin; *Eco*RI treatment allowed 92% of the excised origin to elute but only 7% of the rest of the plasmid (longer exposures of lanes 8,10; not shown). The partial-digestion pattern given by *Bgl*I is more complex, but still allows individual, or closely related, fragments to be identified and their retentions compared (lanes 6,12); in this and different experiments (not shown), bands containing the largest *Bgl*I fragment (i.e. containing the globin transcription unit) were three to six times more enriched than other bands in the attached fraction. After shredding minichromosomes with *Hae*III, only 8% of the resulting ~400 bp fragments were retained (lane 13; Table 2).

As Fig. 2 shows that most intact minichromosomes are retained, but so few derivative fragments that each minichromosome must be attached at few points. The precise number was deduced - using the method described by Jackson et al. (1990a) - from the percentage of plasmid DNA remaining, the sizes of attached fragments and the initial length of the plasmid: plasmids were attached at 1-2 points (Table 2).

Transcription units are attached

Attached regions were mapped as follows. Beads were incubated with *Hae*III, half were subjected to electrophoresis and DNA was purified from the two halves. DNA from each - and from known numbers of cell-equivalents - was then 'slot-blotted' onto 10 identical filters and probed with ^{32}P-labelled regions 1-10 (Fig. 1); then 10 autoradiographs were prepared (Fig. 3A; relative loadings are given at bottom left). The percentage of each region that remained attached was then determined by quantitative densitometry (Fig. 3B). Between 10 and 15% cellular fragments resisted elution, but only 5-8% of minichromosomal fragments, probably reflecting the high density of *Hae*III sites on the minichromosome. Promoters (i.e. regions 2 and 5) and other parts of the two transcription units (i.e. regions 1,10 and 6,7) were the least depleted. Significantly, the relative retentions of the two transcription units reflects their activities (see later). Note that *Hae*III fragments are not retained according to their size; the largest fragment (covering most of region 3) is the most depleted and, conversely, the most highly retained region (i.e. 5) contains many of the smallest fragments (Fig. 1).

Less than 3% of pBR322 sequences (i.e. regions 3,4,9) remained (Fig. 3B). Most of them probably reflect true attachments that become noticeable only after removing most of the chromatin, rather than non-specific (i.e. background) binding. Thus, although most fragments are ~400 bp (Table 2), 1-2% (by weight) are partial-digestion products, >2 kb; after elution these constitute 10-20% of retained fragments. Then pBR322 sequences at one end of such large fragments will be attached through transcribed regions at the other. The minority of replication-mediated attachments also becomes more significant after elution (Jackson and Cook, 1986).

Attached regions are associated with active polymerases

The preferential retention of transcription units suggests that active polymerases mediate attachments. We confirmed that active transcription complexes were associated with retained fragments by performing a 'run-on' analysis; the number of transcripts made in vitro was similar whether or not most minichromosomal chromatin had been removed.

We first established the necessary conditions. Transfected cells were encapsulated, permeabilized and incubated with [^{32}P]UTP to elongate existing nascent transcripts. A low

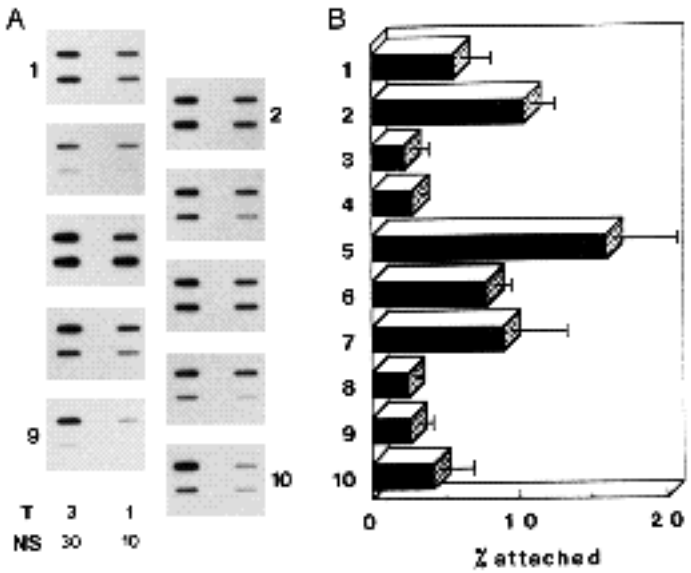

Fig. 3. Promoters and transcribed regions are preferentially attached. (A) Transfected cells were labelled with [^{3}H]thymidine, encapsulated, lysed, incubated with *Hae*III (500 units/ml) and half were subjected to electrophoresis, DNA was purified from both halves and the ^{3}H content was determined; 10 identical filters were prepared by slot-blotting [^{3}H]DNA from the 2 samples as illustrated at the bottom left (T, total DNA from beads not eluted; NS, nucleoskeleton-associated DNA from eluted beads that retained 11% chromatin; numbers indicate relative loadings of cell equivalents). Filters were probed with [^{32}P]regions 1-10 (Fig. 1); the resulting autoradiographs (4 h exposure) are shown. Relative signals given by DNA from equivalent cell numbers reflects the % of a region that remains attached after elution. (B) Signal intensities were determined by densitometry or scintillation counting of autoradiographs like those in A. The % of *Hae*III fragments remaining attached that hybridized with regions 1-10 is shown (av. of 3 experiments (+ s.d.), in which 10-15% ^{3}H resisted elution).

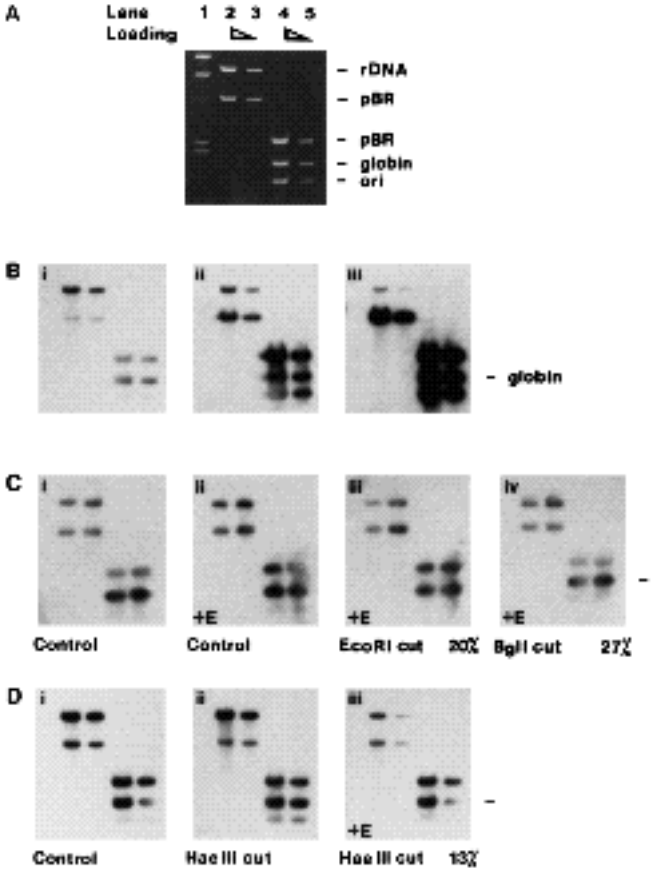

Fig. 4. Engaged polymerases resist elution. (A) Ten identical gels were run, stained with ethidium and blotted to give the 10 filters used in (B-D); a photograph of the relevant region of one gel is shown. Lane 1, lambda/*Hin*dIII. Lanes 2, 3, pMS5/*Eco*RI. Lanes 4, 5, pSVα1fd cut with *Bam*HI, *Sca*I and *Pst*I. Lanes 1, 2, 4 and 3, 5 were loaded with 1 and 0.33 μg DNA, respectively. pBR322, human rDNA, α-globin and SV40 *ori* fragments are indicated; the globin fragment is also indicated on the right in (B-D). (B) Cells were transfected (i-iii: 1, 5, 25 μg/flask), harvested after 42 h, encapsulated (5×10^6 cells/ml), permeabilized, precursors added and [^{32}P]UTP incorporated into RNA in vitro. RNA from the 3 samples was hybridized with 3 filters and autoradiographs prepared (1 week exposure). (C) As (Bi) except that beads were treated without (control) or with *Eco*RI or *Bgl*I and some were eluted (+ E). The % chromatin remaining is given in iii and iv. (D) As (C) using *Hae*III.

triphosphate concentration was deliberately used to limit chain extension to ~30 nucleotides. [^{32}P]RNA made in vitro was then degraded to 75-150 nucleotides, purified and hybridized with several identical filters prepared by blotting various DNA targets (Fig. 4A). Autoradiographic signals then reflect the concentration of ^{32}P-labelled transcripts and so the number of polymerases associated with particular sequences (Fig. 4B-D). (Under these conditions hybridization is filter-driven, as indicated by roughly similar signals given by threefold differences in filter-bound target (in each case, compare lanes 2 with 3 and 4 with 5).) The labelled transcripts hybridized with rDNA, the globin gene and pBR322 sequences transcribed by 'read-through' from the SV40 promoter, but not with lambda sequences (Fig. 4Bi). When signal intensities are corrected for target length, the globin promoter proves to be two to threefold more active than the viral promoter, reflecting the relative retentions seen earlier. After transfecting higher inputs (Fig. 4Bi-iii), cellular rDNA signals (i.e. given by the top band) remained roughly constant as the same number of polymerases are transcribing the fixed number of ribosomal cistrons (i.e. in Fig. 4Bi-iii, the intensities of the top band are roughly equal). In contrast, plasmid signals become stronger as the number of active minichromosomes increases with the percentage of transfected cells (i.e. in Fig. 4Bi-iii intensities of the 4 lower bands increase). These controls confirm that signal intensities reflect polymerase numbers associated with different fragments. Experiments described elsewhere show that most of this 'run-on' transcription is sensitive to 10 μg/ml α-amanitin and so due to RNA polymerase II (e.g. Fig. 5 of Dickinson et al., 1990).

Fig. 4C,D shows that active polymerases are almost exclusively associated with residual fragments; even when most of the chromatin is removed, the signals given by the different bands are all roughly the same. For example, *Hae*III cutting and elution removed 87% chromatin and 94% plasmid DNA but had little effect on signals given by any

of the bands (Fig. 4Di-iii). Clearly, active polymerases transcribing both cellular rDNA (top band) and minichromosomes (bottom 4 bands) are retained, even when most of the chromatin is removed. This was confirmed by measuring total elongating activity before and after electrophoresis as described by Dickinson et al. (1990); electrophoresis removed 11% activity (av. of 7 experiments; ± s.d. = 7%) and cutting plus electrophoresis removed 70-91% cellular chromatin but only 19% activity (av. of 15 experiments; ± s.d. = 9%). (As polymerizing activity fails to elute at a different pH (i.e. pH 8.0), the failure cannot result from a fortuitous lack of charge on the polymerizing complex in the physiological buffer (Jackson and Cook, 1985b).)

To eliminate the possibility that active polymerases failed to elute because of some property of their associated RNA - perhaps because it had an extended conformation or was non-specifically aggregated - the experiments in Figs 2 and 4C were repeated after removing most nascent RNA as described by Jackson and Cook (1985b). Transfected and encapsulated cells were labelled for 5 min with [^{3}H]uridine immediately before lysis, and then beads were treated with sufficient RNase to remove >95% ^{3}H-labelled (i.e. nascent) RNA prior to elution. Despite removal of nascent RNA, active polymerases still failed to elute (not shown; Dickinson et al., 1990, have studied this point in detail).

Next, the precise distribution of active polymerases was analysed. [^{32}P]RNA made in vitro using beads containing total (T) or only 10% chromatin ('nucleoskeleton-associated', NS) was hybridized with two filters on which were arrayed regions 1-10 (Fig. 5A). Signals again reflect polymerase density in each region. As expected, regions downstream from the two promoters (i.e. 1,2,6,7,8) gave intense signals, whilst regions between the divergent promoters (i.e. 3,4,5) gave low signals (Fig. 5A; T). This pattern persisted even when 90% chromatin was removed (Fig. 5A; NS). The relative polymerizing activity associated with each region was then determined by quantitative densitometry and the averages from three such experiments are given in the histogram (Fig. 5B). Active polymerases are only associated with transcribed regions (hatched bars) and removing most chromatin removes few of them (filled bars). Some loss is to be expected, of course, because the template is so truncated. Significantly, active polymerases are bound to fragments shown to be retained preferentially in Fig. 3. Note also that few transcripts hybridized with promoter region 5, which was retained in Fig. 3B; it must be attached through some mechanism other than one involving an elongating polymerase.

The authenticity of these transcripts was confirmed by hybridizing the two types of [^{32}P]RNA to filter-bound single strands (both + and −) from around the globin locus (Fig. 1). Labelled RNA hybridized only with the transcribed globin strand (Fig. 5C, strand c) but not flanking or non-transcribed strands. This was so even when most chromatin (whether cellular or plasmid) had been removed (Fig. 5D, cf. hatched and filled bars). Clearly, transcripts made in vitro are authentic, with little synthesis initiating at ends generated by *Hae*III digestion.

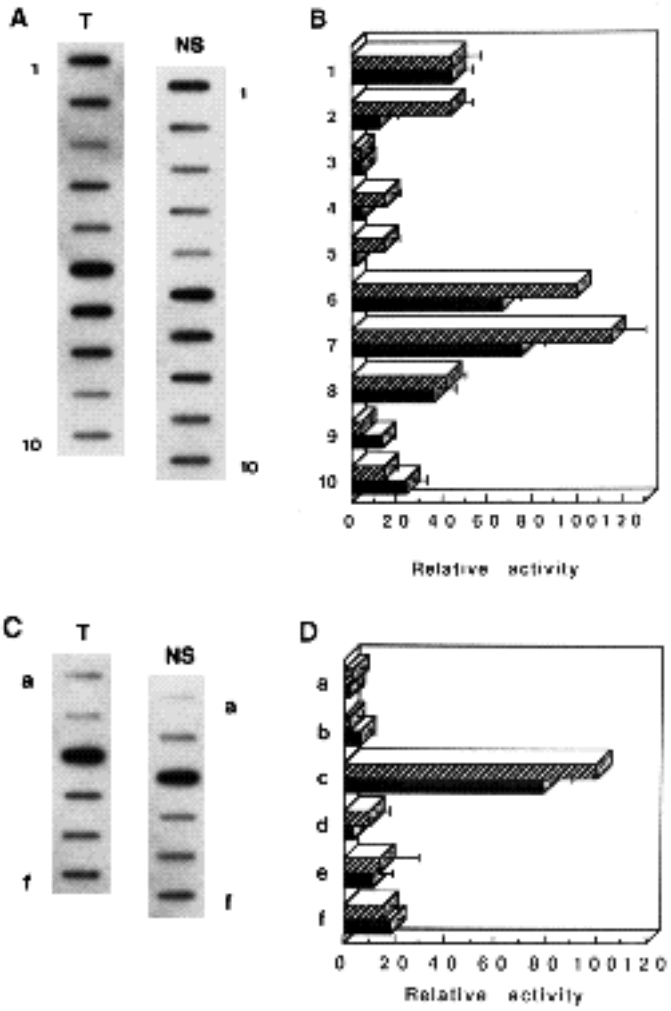

Fig. 5. Differential retention of active polymerases.
(A) Permeabilized cells were treated ± *Hae*III and half were electroeluted. [^{32}P]RNA was made in vitro from beads that retained 100% (T) or 10% chromatin (i.e. nucleoskeleton-associated; NS), hybridized to 2 filters (2.5×10^6 c.p.m./filter) on which were arrayed 200 ng regions 1-10 and 2 autoradiographs prepared (2 week exposure). (B) Band intensities were then quantified by densitometry and expressed relative to that of the strongest band (i.e. 6), after correcting for region length. Therefore the histogram reflects the relative polymerizing activity associated with each region (average +s.d. of 2 or more experiments). Hatched and filled bars: activities given by beads containing 100 or 10% chromatin, respectively. (C) [^{32}P]RNA made as in (A) was hybridized to 2 filters on which were arrayed 200 ng single-stranded DNA fragments a-f from around the globin gene (Fig. 1). Typical autoradiograms are shown. (D) Relative activities were determined as in (B).

Unattached minichromosomes are not transcribed

These experiments show that attached minichromosomes are transcribed but do not address whether unattached plasmids are not. Experiments like those in Fig. 4Ci,ii were repeated using an input of 25 μg/plate, so generating a population of unattached minichromosomes (Table 1). After elongation in vitro, hybridization of the resulting [^{32}P]transcripts with filter-bound targets and autoradiography, signals again indicate the number of active polymerases associated with each target. Elution removed 70% of

minichromosomes but reduced signals by only 5% (averages of 3 experiments, not shown; again Dickinson et al., 1990, have shown that active polymerases resist elution); only inactive minichromosomes are lost.

DISCUSSION

After transfection into cos7 cells, pSVα1fd replicates over 2 days to give several thousand minichromosomes. These provide simple models for chromatin domains; they support authentic nucleosomal chromatin and their genes are expressed appropriately (e.g. the globin gene yields its natural message; Table 1). We mapped sequences attaching them to larger nuclear structures; after cutting with nucleases, attachments were defined operationally by their ability to prevent chromatin fragments electroeluting from nuclei. Elution leaves transcription sites and residual clumps of cellular chromatin attached to an underlying nucleoskeleton (Jackson and Cook, 1988; Jackson et al., 1993), so residual minichromosomal fragments are probably attached, directly or indirectly, to the same skeleton.

There were three major populations of minichromosomes: <5% were replicating at any one time and, of the rest, some were attached and the remainder unattached. The number of minichromosomes per cell could be varied by varying the input. With high inputs, most could be eluted from permeabilized cells; with lower inputs, a constant number (i.e. ~1200/cell) of plasmids resisted elution (Table 1). As few, if any, of the eluted fraction are transcribed, attachment correlates with transcriptional activity.

The number of attachments

The minichromosomes that resisted electroelution were not non-specifically trapped in dense cellular chromatin as removing it does not allow them to escape (Fig. 2, lane 9). After cutting into ~400 bp pieces, quantitative analysis of fragments resisting elution showed that each plasmid was attached at only 1-2 points (Table 2).

The nature of the attachments

Perhaps surprisingly, no one sequence was responsible for attachment. The multi-functional *ori* is a candidate for an attached region (Schirmbeck and Deppert, 1991), but it was attached in only 10% of bound minichromosomes (Fig. 3B), reflecting the activity of its associated transcription unit. Note also that neither the *ori* nor the α-globin gene are attached in nuclear scaffolds, nor do they bind preferentially to matrices (i.e. they are not SARs or MARs; Pommier et al., 1990; Jarman and Higgs, 1988).

Minichromosomes are attached almost entirely through promoters and their associated transcription units, with the degree of attachment reflecting the unit's activity (Figs 3,4); this suggests that polymerases are responsible for attachments in transcribed regions. Both transcription units are transcribed by RNA polymerase II as they can be inhibited by 10 μg/ml α-amanitin (not shown; Dickinson et al., 1990). As there were few elongation-competent polymerases on the globin promoter (Fig. 5A,B), it must be attached in another way, perhaps through transcription factors or a bound polymerase that has not yet initiated. If minichromosomes are attached in vivo through other sequences, then these attachments must be disrupted during lysis and/or elution.

Several reasons make it unlikely that transcribed regions fail to elute because they have aggregated artifactually. (i) Hypotonic treatments 'precipitate' active chromatin so it resists nucleolytic removal (Rose and Garrard, 1984; Stratling et al., 1986; Delcuve and Davie, 1989) but >90% of our active minichromosomal chromatin elutes after *Hae*III cutting and the remainder is attached specifically. (ii) Artifactual aggregation should inhibit polymerization, but transcription continues - under optimum conditions - authentically along the correct strand (Fig. 5D) at roughly the in vivo rate (Jackson et al., 1988). (iii) Polymerizing activity still fails to elute after removing >95% nascent RNA with RNase (see Results and Dickinson et al., 1990) when any attachments through transcripts should be destroyed. (iv) As active complexes are associated with chromatin fragments of various sizes, it seems improbable that all would have no net charge and so fail to elute at both pH 7.4 (used here) and pH 8.0 (used by Jackson and Cook, 1985b). (v) If a complex contains ~100 polypeptides of ~50 kDa, it would still only be the size of a minichromosome, a structure that can elute (Table 1).

Recent results show that active RNA polymerases are not diffusely spread throughout euchromatin, but concentrated in ~300-500 focal sites that resist elution; each site contains ~60 polymerases (Jackson et al., 1993; Xing et al., 1993). Such transcription foci are probably analogous to the replication 'factories' seen by electron microscopy; these are large ovoid structures (diam. 175 nm) attached to a skeleton, with each containing ~40 active replication forks (Hozák et al., 1993). Then our results are most simply explained if transfected plasmids can only be replicated or transcribed by incorporation into the appropriate factory.

Transient and dynamic attachments

We envisage that transcriptionally active minichromosomes are attached transiently in a factory at only one to two points, through a promoter or a transcription unit. At any moment individual templates will be at different stages in a cycle: some will have just attached at a promoter, others will be attached at different points along the template as it slides past the static enzyme, still others will have detached and become inactive. No one point is always attached; different points have different probabilities of attachment. According to this view, the polymerase specifies an attachment point, playing both a structural and a functional role as the key component of a solid phase on which RNA synthesis, processing and transport all take place (reviewed by Cook, 1989; Carter et al., 1991).

We thank Emma Whitelaw for the M13 clones, Mike Simpkins and Sandra Smith for their help, and the Cancer Research Campaign for support.

REFERENCES

Bonnerot, C., Grimber, G., Briand, P. and Nicolas, J.-F. (1990). Patterns of expression of position-dependent integrated transgenes in mouse embryo. *Proc. Nat. Acad. Sci. USA* **87**, 6331-6335.

Briggs, D., Jackson, D., Whitelaw, E. and Proudfoot, N. J. (1989). Direct demonstration of termination signals for RNA polymerase II from the sea urchin H2A histone gene. *Nucl. Acids Res.* **17**, 8061-8071.

Carmo-Fonseca, M., Pepperkok, R., Sproat, B. S., Ansorge, W., Swanson, M. S. and Lamond, A. I. (1991a). In vivo detection of snRNP-rich organelles in the nuclei of mammalian cells. *EMBO J.* **10**, 1863-1873.

Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S. M. L., Merdes, A., Brunner, C., Zamore, P. D., Green, M. R., Hurt, E. and Lamond, A. I. (1991b). Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO J.* **10**, 195-206.

Carter, K. C., Taneja, K. L. and Lawrence, J. B. (1991). Discrete nuclear domains of poly(A)RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.* **115**, 1191-1202.

Charnay, P., Treisman, R., Mellon, P., Chao, M., Axel, R. and Maniatis, T. (1984). Differences in human α- and β-globin gene expression in mouse erythroleukemic cells: the role of intragenic sequences. *Cell* **38**, 251-263.

Cook, P. R. (1988). The nucleoskeleton: artefact, passive framework or active site? *J. Cell Sci.* **90**, 1-6.

Cook, P. R. (1989). The nucleoskeleton and the topology of transcription. *Eur. J. Biochem.* **185**, 487-501.

Delcuve, G. P. and Davie, J. R. (1989). Chromatin structure of erythroid-specific genes of immature and mature chicken erythrocyte. *Biochem. J.* **263**, 179-186.

Dickinson, P., Cook, P. R. and Jackson, D. A. (1990). Active RNA polymerase I is fixed within the nucleus of HeLa cells. *EMBO J.* **9**, 2207-2214.

Hozák, P., Hassan, A. B., Jackson, D. A. and Cook, P. R. (1993). Visualization of replication factories attached to a nucleoskeleton. *Cell* **73**, 361-373.

Huang, S. and Spector, D. L. (1991). Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes Dev.* **5**, 2288-2302.

Jackson, D. A. (1991). Structure-function relationships in eukaryotic nuclei. *BioEssays* **13**, 1-10.

Jackson, D. A. and Cook, P. R. (1985a). A general method for preparing chromatin containing intact DNA. *EMBO J.* **4**, 913-918.

Jackson, D. A. and Cook, P. R. (1985b). Transcription occurs at a nucleoskeleton. *EMBO J.* **4**, 919-925.

Jackson, D. A. and Cook, P. R. (1986). Different populations of DNA polymerase α in HeLa cells. *J. Mol. Biol.* **192**, 77-86.

Jackson, D. A. and Cook, P. R. (1988). Visualization of a filamentous nucleoskeleton with a 23 nm axial repeat. *EMBO J.* **7**, 3667-3677.

Jackson, D. A., Dickinson, P. and Cook, P. R. (1990a). The size of chromatin loops in HeLa cells. *EMBO J.* **9**, 567-571.

Jackson, D. A., Dickinson, P. and Cook, P. R. (1990b). Attachment of DNA to the nucleoskeleton of HeLa cells examined using physiological conditions. *Nucl. Acids Res.* **18**, 4385-4393.

Jackson, D. A., Hassan, A. B., Errington, R. J. and Cook, P. R. (1993). Visualization of focal sites of transcription within human nuclei. *EMBO J.* **12**, 1059-1065.

Jackson, D. A., Yuan, J. and Cook, P. R. (1988). A gentle method for preparing cyto- and nucleo-skeletons and associated chromatin. *J. Cell Sci.* **90**, 365-378.

Jarman, A. P. and Higgs, D. R. (1988). Nuclear scaffold attachment sites in the human globin gene complexes. *EMBO J.* **7**, 3337-3344.

Lawrence, J. B., Singer, R. H. and Marselle, L. M. (1989). Highly localized tracks of specific transcripts within interphase nuclei visualised by in situ hybridization. *Cell* **57**, 493-502.

Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. (1981). Identification of DNA sequences required for transcription of the human α1-globin gene in a new SV40 host-vector system. *Cell* **27**, 279-288.

Myers, R. M. and Tjian, R. (1980). Construction and analysis of simian virus 40 origins defective in tumour antigen binding and DNA replication. *Proc. Nat. Acad. Sci. USA* **77**, 6491-6495.

Pommier, Y., Cockerill, P. N., Kohn, K. W. and Garrard, W. T. (1990). Identification within the simian virus 40 genome of a chromosomal loop attachment site that contains topoisomerase II cleavage sites. *J. Virol.* **64**, 419-423.

Rose, S. M. and Garrard, W. T. (1984). Differentiation-dependent chromatin alterations precede and accompany transcription of immunoglobulin light chain genes. *J. Biol. Chem.* **269**, 8534-8544.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning, a Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Sanes, J. R., Rubenstein, J. L. R. and Nicolas, J.-F. (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5**, 3133-3142.

Schirmbeck, R and Deppert, W. (1991). Structural topography of SV40 DNA replication. *J. Virol.* **65**, 2578-2588.

Stratling, W. H., Dolle, A. and Sippel, A. E. (1986). Chromatin structure of the chicken lysozyme gene domain as determined by chromatin fractionation and micrococcal nuclease digestion. *Biochemistry* **25**, 495-502.

Xing, Y., Johnson, C. V., Dobner, P. R. and Lawrence J. B. (1993). Higher level organization of individual gene transcription and RNA splicing. *Science* **259**, 1326-1330.

(Received 21 April 1993 - Accepted 19 May 1993)