## Attachment of repeated sequences to the nuclear cage

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#### ABSTRACT

Nuclear DNA is probably organized into loops by attachment to a sub-structure in vivo. When HeLa cells are lysed in Triton and 2M NaCl the resulting nucleoids contain naked DNA which is supercoiled so the loops must remain intact. We have attempted to identify sequences responsible for attaching these loops to the nuclear sub-structure by progressively detaching DNA with various nucleases. Fragments at the 5' end of the ribosomal RNA locus, and a variety of transcribed and repeated sequences, are shown to lie relatively close to attachment points. This implies that sequences cannot be arranged randomly. However no "attachment sequence" could be identified.

#### INTRODUCTION

It is now widely believed that nuclear DNA is looped by association with a nuclear sub-structure, the matrix or cage. This belief stems in part from the striking images of loops of DNA in lampbrush or mitotic chromosomes (1), the demonstration of supercoiling in interphase DNA (2) and the kinetics of digestion of the DNA in rat liver nuclei by nucleases (3). Since the existence of independent DNA domains could have important consequences on nuclear structure and function, the mechanism by which they are formed is of interest. The position of genes within such DNA domains or loops may be analysed by 'detachment mapping' (4). Nucleoids, containing intact DNA arranged in loops of approximately 220 kbp (2,5) are incubated with a restriction endonuclease. Following digestion, the sub-structures and their associated DNA are separated from any detached DNA fragments by centrifugation. Clearly, a gene positioned such that there are many restriction sites between it and the sub-structure is more likely to be detached than another gene with fewer such interstitial sites. If a gene is similarly positioned in all the cells of a population it will either be enriched or depleted in the pelleted fraction, depending on this position. Different degrees of

enrichment should be seen as different amounts of DNA are detached. Alternatively, if the gene is randomly associated with the sub-structure in the cell population it will neither be enriched nor depleted.

Using this technique Cook and Brazell (4) found that globin sequences were positioned specifically within the HeLa cell nucleus. Subsequently, this type of analysis has been extended to other genes. For example, integrated viral sequences (6,7) the ovalburnin gene (8-10) and some cellular repeats (11-14) are enriched in the pelleted fraction. However, convincing enrichments are not found in all cases (15,16). Decisive evidence for specific attachment would be provided by the isolation of "attachment sequences". If such sequences maintained DNA loops through their association with the nuclear sub-structure they might resist detachment by nucleases. They would be moderately repeated in total DNA.

However, analysis of sequences resisting digestion with nucleases has yielded conflicting reports. Using solution hybridization Razin <u>et</u> <u>al</u>. (17,18) and Jeppesen and Bankier (19) found repeats enriched in the resistant DNA whilst others found this residual DNA to be equivalent to bulk DNA (12,20). Repetitive sequences are clearly associated preferentially with the sub-structure at certain levels of digestion (11,12,19) but to date no sequences which behave as "attachment sequences" have been identified.

In this paper we describe the characterization of DNA attachments within HeLa nucleoids. We began by studying attachment within a wellcharacterized repeat, the ribosomal RNA locus. Using various restriction endonucleases we confirm that ribosomal sequences lie close to the nuclear sub-structure (21) and show that some parts of the locus are more closely associated than others. However, our results are inconsistent with the simple model where only one part of the locus is stably attached. We then attempted to identify attachment sites by analysing sequences resisting digestion by non-specific nucleases. Like others (17-19), we find a variety of repeats enriched in our 'cage-associated' fractions, but none behave as if they are found solely at the base of loops. Instead, they share similarities with attached rDNA sequences.

#### MATERIALS AND METHODS

## Cell growth and synchronization

HeLa cells were grown in suspension (2) and >90% synchronized in

mitosis, where indicated, using nitrous oxide (22,23). Cells in G<sub>1</sub> were obtained 2 h after release of the nitrous oxide block. Isolation of nucleoids and detachment mapping

Nucleoids were isolated by a slight modification of the original technique (24). About 2 x 10<sup>8</sup> HeLa cells were labelled with [methyl-<sup>3</sup>H] thymidine (0.05  $\mu$ Ci/ml for 16 h; 30-60 Ci/mmol) resuspended in 4.5 ml phosphate-buffered saline, lysed by adding 3 vols lysis mix [2.6 M NaCl, 133 mM EDTA, 2.7 mM Tris-HCl (pH 8.0) and 0.67% Triton X-100] and layered on to 3 x 20 ml step gradients of 15% sucrose, 1.95 M NaCl, 10 mM Tris-HCl and 1 mM EDTA floating on 5 ml of 30% sucrose, 1.95 M NaCl, 10 mM Tris-HCl and 1 mM EDTA. After 10 min on ice the sample was spun (5,500 rpm; 25 min; Beckman SW28 rotor; 4°C). The fluffy aggregate of nucleoids on the 30% sucrose shelf was collected and diluted with 10 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub> and 1 mM dithiothreitol to give the required NaCl concentration.

Nucleoids were incubated with restriction endonucleases (Boehringer and Bethesda Research Labs; 15-60 min at 37°C; 15-300 units/ml), the reaction stopped by the addition of EDTA to 10 mM and the sample spun (15,000 rpm; 15 min; Beckman SW28 rotor; 4°C) to pellet cages. The pellet was rinsed carefully with 10 mM Tris-HC1 (pH 8.0) or washed by resuspension and re-pelleting and then resuspended in 10 mM Tris-HC1 (pH 8.0), 2 mM EDTA. DNA was purified from total, cage and supernatant samples by incubation with 1% sarkosyl and 200  $\mu$ g/ml heat-inactivated RNase A (2 h; 37°C) and Proteinase K (Boehringer; 125  $\mu$ g/ml; 12 h; 37°C). After raising the NaCl concentration to 0.3 M the samples were extracted twice with phenol, twice with ether and dialysed against 10 mM Tris-HC1, (pH 8.0) 0.1 mM EDTA. The concentration of the 100% DNA sample was determined optically and the concentrations of the others estimated using their relative radioactivities.

Known amounts of DNA were ethanol-precipitated, completely digested with the desired restriction endonuclease, separated on agarose gels, stained with ethidium bromide, photographed and "blotted" (25) on to nitrocellulose. The blotted filters were baked (14-18 h; 65-72°C) and stored under vacuum at room temperature.

## Residual DNA probes

 $2 \times 10^8$  HeLa nucleoids were incubated in 10 ml 0.2 M NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub> with DNase I (Worthington, 50 µg/ml; 37°C; 4-40 h) and RNase A (50 µg/ml), the digested nucleoids pelleted, washed, and the residual nucleic acid purified as described above. Nucleoids were also digested with micrococcal nuclease (Boehringer, 20  $\mu$ g/ml) and RNase A (50  $\mu$ g/ml) in 200 mM NaCl, 10 mM Tris-HCl (pH 8.0), 2 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub> and treated as for those digested with DNase I. In certain experiments DNase I and micrococcal nuclease were used in combination. Residual nucleic acid (10  $\mu$ g/2x10<sup>8</sup> HeLa nucleoids) isolated as described above was nevertheless 95% RNA. Where necessary, this RNA [with fragments in the range 20-180 bases and a characteristic banding pattern (major bands are 180, 120 and 80 bases)] was removed by digestion with RNAse A and RNAse T2.

In some experiments, after pelleting the washed cages were resuspended in 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, heated (96°C; 10 min) and cooled rapidly on ice. The cages were then treated with S<sub>1</sub> nuclease (Sigma, 5000 u/ml; 37°C; 30 min) in 50 mM sodium acetate (pH 4.5) and 2 mM  $2nSO_4$  prior to purification of residual nucleic acid. Some nucleoids treated with DNase and micrococcal nuclease were also centrifuged to equilibrium in CsCl gradients  $\pm 2.5$  M guanidinium hydrochloride or 1% Sarkosyl (final density 1.75 or 1.30 g/ml; Beckman SW 50.1; 72 h; 35000 rpm; 25°C), and nucleic acid prepared from fractions containing cages (at 1.24-1.28 g/ml CsCl).

Nick-translation and end-labelling

DNA was 'nick-translated' (26) to a high specific activity (2-8 x  $10^{8} \text{ dpm}/\mu g$ ) using a nick-translation kit (Amersham) and  $[\alpha - 3^{2}P]$ dATP (Amersham; >2000 Ci/mmol; 10  $\mu$ Ci/10 ng DNA; 1.5 h; 17°C). DNA and RNA were end-labelled (27) in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub> and 1 mM dithiothreitol using polynucleotide kinase (Boehringer; 5 u/10 ng nucleic acid) and  $[\gamma - 3^{2}P]$ ATP (Amersham; >2000 Ci/mmol; 10  $\mu$ Ci/10 ng nucleic acid; 1.5 h; 37°C).

## Hybridization

Nitrocellulose filters were prehybridized (6-16 h; 42°C) in 5 ml 50% formamide, 3x SSC, 3x Denhardt's solution and 500  $\mu$ g/ml yeast tRNA and hybridized (18-40h; 42°C) in 2.5 ml of the above containing <10 ng of [<sup>32</sup>P]DNA. Filters were rinsed three times in 200 ml 2x SSC, 0.1% SDS (20 min; 20°C) then twice in 200 ml 0.1x SSC, 0.1% SDS (20 min; 55°C). Autoradiographs were prepared using pre-flashed (28) Fuji RX X-ray Film and Ilford intensifying screens at -70°C.

Hybridization at lower stringencies was carried out as described (29). Nitrocellulose filters were prehybridized (6-16 h; 4 or 20°C) in 2.5 ml 6x SSC, 10x Denhardt's solution and hybridized (2-12 h; 4 or 20°C) in 0.75 ml



Fig. 1. A map of the human ribosomal RNA locus (37-39). Regions coding for the 18 and 28S ribosomal RNA are represented by the thick lines. The Xenopus rDNA probe hybridizes with the underlined sequence. EcoRI (●), and <u>Hind</u> III (●) digestion sites are indicated. Many <u>Hae</u> III sites are scattered throughout the locus. The restriction fragments labelled a-f (kbp) are referred to in the text.

of the above with 0-40% formamide and <5 ng of  $[^{32}P]DNA$ . Filters were washed extensively in 6x SSC, 10x Denhardt's solution at the hybridization temperature, and re-washed subsequently as above.

Prior to rehybridization, nitrocellulose filters (stored wet in bags at 4°C after autoradiography) were washed (30 min; 30°C) in 90% formamide, 0.1% SDS and 0.1x SSC to remove old probe.

## RESULTS

## Attachment of rDNA

Fig. 1 illustrates a map of the human ribosomal RNA repeat. The positions of the 28 and 18S ribosomal RNA genes relative to the nuclear cage were mapped as shown in Fig. 2. Total DNA (100% remaining) and the 6% of the DNA which pelleted with the nuclear cage following partial  $\underline{Eco}$  RI digestion were purified, completely redigested with  $\underline{Eco}$  RI, various amounts separated in an agarose gel and then blotted. Hybridization with a rDNA probe reveals bands of 5.7, 7.3 and 20 kbp (Fig. 2, channels 1-3). The signals from the cage-associated sample are >5x more intense than those obtained with an equal weight of total DNA (compare channels 2 and 3). The enrichments obtained with a range of different levels of detachment are listed in Table 1. When <u>Hind</u> III replaces <u>Eco</u> RI in both digestions the resulting 13 and 16 kbp rDNA fragments are similarly enriched (Fig. 2, channels 4-6).

Differential digestion of various  $\underline{\text{Eco}}$  RI sites is revealed by the following variation of the basic mapping procedure. The DNA samples used for Fig. 2, channels 1-3 were completely recut with <u>Hind</u> III, rather than  $\underline{\text{Eco}}$  RI (Fig. 2, channels 7-9). Complete digestion of pure



Fig. 2. Detachment mapping ribosomal genes in HeLa nucleoids. Total DNA (100%; channels 1,2,4,5,7,8) and DNA that resists detachment by a restriction endonuclease (200 u/ml; 15 min) from the cage (6% and 8%; channels 3,6,9) were purified, redigested completely and applied to a 0.8% agarose gel. Following electrophoresis, blotting, and hybridization with the rDNA probe an autoradiograph was prepared. In channels 1-3, both digestions were with Eco RI, in channels 4-6, both were with Hind III and in channels 7-9 the first was with Eco RI and the second Hind III. The positions of marker fragments of XDNA cut with Hind III are indicated. 1x DNA is 1 μg. a-f are referred to in the text.

total DNA with both enzymes yields 3 rDNA bands; a 5.7 kbp  $\underline{\text{Eco}}$  RI fragment, a 7 kbp  $\underline{\text{Eco}}$  RI-<u>Hind</u> III fragment (Fig. 1, fragments a,b) and a 0.3 kbp  $\underline{\text{Eco}}$  RI-<u>Hind</u> III fragment. An initial partial  $\underline{\text{Eco}}$  RI digestion followed by complete digestion with <u>Hind</u> III should yield the same 3 rDNA fragments and four additional ones (Fig. 1, fragment c-f) containing uncut  $\underline{\text{Eco}}$  RI sites. Irrespective of the extent of the initial  $\underline{\text{Eco}}$  RI digestion, the <u>relative</u> intensity of the hybridization signals to the large or small fragments should remain constant (i.e. e:d+f and b:a+c = constant) if the  $\underline{\text{Eco}}$  RI sites E1-E4 (Fig. 1) are digested to the same extent in nucleoids. In Fig. 2, channels 1 and 2, where all  $\underline{\text{Eco}}$  RI sites have been cut, the relative intensity of the 7.3 to 5.7 kbp fragment (i.e. effectively b:a+c where c = 0) is 1:0.26. Variations in this Table 1. Enrichment of rDNA following detachment with Eco RI. Autoradiographs like those in Fig. 2 were scanned using a Joyce-Loebl densitometer and enrichments of the various fragments estimated from the relative peak areas. Samples marked \* were treated with 0.08  $\mu$ g/ml actinomycin D for 1 h prior to harvesting cells. Analysis of the corresponding cage-released fragments confirmed enrichment of rDNA sequences in the cage-associated DNA.

\$ DNA remaining		Enrichmer	nt ( <b>%</b> r	DNA re	covered	with	cage)	
with cage	Partia	lly-digested	20 kbp		7.3 kbp		5.7 kbp	
	Fr	agments						
32	3x	(100%)						
13	6x	(785)						
11	5x	(55%)						
10	7x	(70%)						
8			5.0x	(40%)	7.0x	(56%)	6.0x	(48%)
6	9x	(54%)			•			
6			3.2x	(20%)	7.5x	(45%)	9.0x	(54%)
5			3.0x	(15%)	5.0x	(25%)	6.0x	(30%)
5			3.0x	(15%)	4.5x	(23%)	5.0x	(25%)
5#			3.0x	(15%)	4.5x	(23%)	5.0x	(25%)
2.5			2.0x	(5%)	6.0x	(15%)	7.0x	(18%)
2*			2.5x	(5%)	4.5x	(9%)	5.5x	(11%)
1.5			<0.5x	(1%)	4.0x	(6%)	5.0x	(8%)
1.5			<0.5x	(1%)	4.8x	(7%)	8.0x	(12%)

relative intensity obtained after partially digesting <u>nucleoid</u> DNA reflect differences in the frequency of <u>Eco</u> RI cutting at the various sites. Thus, in channels 7 and 8, the 6 kbp bands (a+c) are very faint and the 13 kbp bands (d+f) are correspondingly darker: the relative intensity (b:a+c) has fallen to 1:0.03. Since no such variation in relative intensity is seen when partial digestion experiments are performed on pure DNA (results not shown) this implies that <u>Eco</u> RI site E2 in nucleoids is relatively less accessible to <u>Eco</u> RI than site E4. (Accessibility of site E3 cannot be ascertained by this method, since <u>Hind</u> III cuts too close to it). Moreover, the 6 kbp fragment is enriched 15x in the cage sample (Fig. 2, compare channels 7 and 9) suggesting that this 5' fragment is most closely associated with the nuclear sub-structure.

If any rDNA fragment is attached, its greatest enrichment should be seen when the maximum amount of DNA is detached. Therefore, HeLa nucleoids were digested with  $\underline{\text{Eco}}$  RI for 48 h to detach as much DNA as possible: the 1.5% of the DNA remaining attached was purified and applied to a gel without further digestion. A photograph of the stained gel shows the distribution of fragments in both total DNA and the cageassociated fraction to be typical of a complete digestion (Fig. 3a).

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Fig. 3. Association of rDNA with the cage after limit digestion. Nucleoids were incubated with Eco RI (50 u/ml) for 48 h. Total
DNA (100%) was purified from one sample; an identical sample was spun and the DNA (1.5%) that pelleted with the cages purified also. Both samples were subjected to electrophoresis in a 0.8% agarose gel (1x is 1 µg), stained with ethidium, photographed (a), blotted, hybridized and an autoradiograph prepared (b; 2 h exposure). Longer exposure revealed no partial digestion products.

Furthermore, no partial digestion products are found in the autoradiograph (Fig. 3b). All the  $\underline{\text{Eco}}$  RI sites must be accessible in nucleoids even though site E2 may be relatively inaccessible. As before, 2 major fragments are detected by the probe. Both are enriched in the cage-associated fraction, the enrichments being 8x and 4.8x for the 5.7 and 7.3 kbp fragments respectively. The third 20 kbp fragment is depleted(results from a longer exposure of the same autoradiograph). If there was one attachment site per ribosomal locus, on complete digestion a band would be either completely detached and so depleted or completely resistant to detachment and so be enriched >50x. The intermediate level of enrichment of both fragments may imply that both are attached in some loci and unattached in others. As the 5.7 kbp is enriched more than the 7.3 kbp fragment, presumably the former is attached more frequently.



Probe; rDNA

Fig. 4. Detachment of rDNA by Hae III.

Nucleoids were incubated with Eco RI (100 u/ml; 15 min) and divided into three. Total DNA (100%; channels 1,2) was purified from one: the 6% of the DNA that cosedimented with cages was purified from the second (6%; channels 3,4). The third sample was adjusted to 10 mM EDTA, pelleted, rewashed to remove Eco RI, incubated with Hae III (100 u/ml; 15 min) and spun; DNA was purified from the pellet (1%; channels 5,6) and the supernatant (channels 7,8). Without further digestion the four DNA samples were subjected to electrophoresis in 1.5% agarose. Photographs of the resulting stained gel (a) and autoradiograph following hybridization with the rDNA probe (b) are shown. The markers (channel m) are  $\lambda$  DNA restricted with Hind III. 1x is 1 µg DNA.

Attachment within the cage-associated fragments was mapped more finely using <u>Hae</u> III, a restriction enzyme with a tetranucleotide recognition sequence (Fig. 4). First, nucleoids were prepared and all but 6% of their DNA detached with <u>Eco</u> RI. The resulting fragments yield a pattern typical of partial digestion on electrophoresis (Fig. 4a, channels 1-4). The autoradiograph confirms that few ribosomal sequences have been cut to 5.7 or 7.3 kbp fragments: the majority run together at the top of the gel (Fig. 4b, channels 1-4). Nevertheless rDNA is clearly enriched >9x, confirming earlier results. Next, these cages with 6% of the total DNA attached were washed free of <u>Eco</u> RI and reincubated with <u>Hae</u> III. After



Fig. 5. The complexity of repeated sequences in fractions resisting detachment by deoxyribonuclease.

Samples of total DNA (100%) and DNA that resisted detachment by Eco RI (11% in a, 13% in b, 2% in c and d, channels 2,3) or Eco RI and Hae III (0.2% in d, channels 4,5) were purified, completely recut with Eco RI (a,b,c) or Hae III (d), subjected to electrophoresis in 0.8% (a,b,c) or 2% (d) agarose, blotted, hybridized with 'nick-translated' probes and autoradiographs prepared. Probes were prepared from 100% HeLa DNA (a) or the residual DNA following detachment of as much DNA as possible with DNase I from cages derived from randomly-growing cells (b,c) or G1 cells (d). The molecular weights are from  $\lambda$  DNA restricted with Hind III (on the left - a,b,c) and PM2 DNA restricted with Hae III (on the right - d). 1x DNA is 3 µg.

incubation, only 1% of the DNA pelleted with cages, the rest was detached and remained in the supernatant. The majority of the pelleted DNA was <2 kbp (Fig. 4a, channels 5,6) and contained little rDNA (Fig. 4b, channels 5,6). Instead most ribosomal fragments (0.1 to 0.6 kbp) were found in the supernatant (Fig. 4b, channels 7,8), indicating that no <u>Hae</u> III fragment detected by our probe contains a unique attachment site. [Note, however, that many fragments are nevertheless attached at a low frequency, consistent with the <u>Eco</u> RI results].

## Attachment of other repeated sequences

In principle, attachment sequences can be purified by completely digesting nucleoid DNA with deoxyribonuclease I (DNase I) to leave only the attached - and so protected - DNA. Less than 0.1% of the total pellets with such DNase-treated cages. There is little contamination of the pelleted DNA with unattached fragments - exhaustive washing does not reduce the recovery below 0.05%. [Washing procedures include repeated resuspension and pelleting, as well as banding in caesium chloride gradients, alone or in the presence of 1% sarcosyl or 2.5 M guanidinium hydrochloride (see materials and methods)]. After purification, electrophoresis and staining, no fragments of discrete size are visible as would be expected of a protected and repeated attachment sequence: instead the fragments (weight-average 100 bp) range in size from 20-500 bp with >50% lying between 50-150 bp.

Sequences complementary to this residual DNA (see Fig. 6c, channel 1) were analysed by 'nick-translating' and hybridizing it with total DNA cut with Eco RI (Fig. 5b, channels 1-3). The resulting autoradiographic smear shows hybridization with a range of Eco RI fragments, especially to 2 of 8.0 and 7.4 kbp and a number <2 kbp. This probe hybridizes to about the same extent with cage-associated DNA (13% remaining), the 8.0 and 7.4 kbp bands being enriched > 4x (Fig. 5b; channels 4,5). When the DNA on the filter was cut with Bam HI, a single band of about 16 kbp was enriched 4x in the cage-associated fraction (10% remaining), while cutting with Hind III yielded 3 enriched bands of 10.1, 8.9 and 6.2 kbp (results not shown). These major enriched bands were shown subsequently to hybridize with mitochondrial DNA. For comparison, Fig. 5a shows the autoradiograph of a similar filter probed with total HeLa DNA, labelled by 'nick-translation'.

Further purification of the attached sequences was attempted using  $S_1$  nuclease. The fact that supercoiling in nucleoid DNA remains after heating nucleoids to 96°C (5) implies that attachments might also be stable under these conditions. Thus, if DNase-digested nucleoids are so treated, on cooling, the attached fragments should renature with zero-order kinetics while non-specifically associated contaminating fragments might renature much more slowly and so be digestible by a single-strand specific nuclease. Hence, after completely digesting nucleoid DNA with DNase I, cages were collected, heated for 10 min at 96°C, cooled rapidly, incubated with  $S_1$  nuclease and the cages repelleted. DNA was purified from the pellet, labelled and hybridized as before to total and cage-associated DNA. However, this probe gave essentially the same hybridization pattern as those obtained without  $S_1$  treatment (results not shown).

Repeated sequences are clearly present in the cage-associated DNA. The repeat complexity of DNA which remained cage-associated following <u>Eco</u> RI digestion was investigated using restriction endonucleases with a four base-pair recognition sequence. For example, DNA that remained cage-associated after detaching 94% of the total reveals a number of repeats upon digestion with <u>Hae</u> III (see also ref 30). Densitometry of an ethidium-stained acrylamide gel (not shown) showed some of these to be enriched and others depleted relative to their counterparts in total DNA. [Enrichments varied from >5x (980 bp repeat), 2-5x (1900, 1300, 860,680, 610, 520 215 bp repeats), 1x (350,330, 100 bp) to <1x (175 bp)]. The enriched bands are seen most clearly when 5-15% of the total DNA remains cage-associated. A less complex pattern is seen as more DNA is detached - when 2% is cage-associated bands of 980, 760, 680, 470 and 325 bp are visible by ethidium bromide staining. The major <u>Hae</u> III repeats (530, 350, 170 and 100 bp) are not enriched significantly at any level of digestion.

In an attempt to simplify our analysis, residual DNA isolated from DNase-treated nucleoids of  $G_1$  cells was used as a probe. Since DNA is replicated at the cage (31) all sequences must become associated with it at some time during S-phase; use of  $G_1$  cells should eliminate this background. After labelling by 'nick-translation' this probe detects a range of repeats in total DNA (Fig. 5d, channel 1) which have a different organization to those seen by ethidium bromide staining. Some of these are slightly enriched (i.e. repeats of 1140,980, 680, 540,460,325, 170 bp) and others depleted (i.e. 1830, 1470 bp) in the cage-associated sample (Fig. 5d; channels 2,3). However, of the enriched fragments all but the smallest cross-hybridize with a mouse mitochondrial DNA probe (unpublished observation). Interestingly, the same repeats are depleted when all but 0.2% of the DNA is detached from nucleoids with Hae III (Fig. 5d, channels 4,5).

A further probe was prepared by completely detaching DNA from cages using micrococcal nuclease. Under our digestion conditions the resistant DNA (about 0.03% of total DNA) is smaller than that obtained with DNase, with a weight average of 35 bp (range 10-200 bp with >50% lying between 10-50 bp). On electrophoresis in an acrylamide gel, 2 bands of 40 and 60 bp are distinguishable, as well as some unresolved bands of smaller size (Fig. 6c and unpublished). After 'nick-translating', this probe hybridized most strongly to a different set of repeats from those detected by our other probe and which do not hybridize with mitochondrial DNA (Fig. 6, compare a with b). As before, no repeat was enriched >10x in the cageassociated fraction (Fig. 6b, channel 4). [End-labelling both probes gave essentially similar results (not shown)].



Probe: DNase limit

M. nuclease limit,

Fig. 6. A comparison of the complexity of repeated sequences in fractions resisting detachment by (a) DNase, (b) micrococcal nuclease.

Samples of total DNA (100%) and DNA that resisted detachment by Eco RI (6% remaining) were purified, completely recut with <u>Hae</u> III, subjected to electrophoresis in 2% agarose, blotted, hybridized with 'nick-translated' probes and autoradiographs prepared. The molecular weight markers are from PM2 DNA restricted with Hae III.

The residual DNA following digestion with DNase (c, channel 1) or micrococcal nuclease (c, channel 2) is visualized following end-labelling. The markers are end-labelled fragments from <u>Alu</u> I restricted ØX174 DNA.

## Association of transcribed genes with the cage

Using solution hybridization, DNA resisting detachment from the cage was found to be enriched in transcribed sequences (32). As others were unable to extend this observation to nuclear matrix DNA (15) this problem was re-examined by hybridizing labelled total cell RNA or poly  $A^+$  RNA (i.e. mRNA) with cage-associated DNA on filters (Fig. 7). Total cell RNA hybridizes with many <u>Hae</u> III fragments in total DNA (Fig. 7a, channels 1,2): the smallest contain rDNA (Fig. 7c, channels 1,2) but we do not know the identities of the others. These must be transcribed repeat sequences, but are not 'Alu' repeats, which are not enriched in this fraction of cage-associated DNA (unpublished results). The rDNA fragments

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# Fig. 7. Transcribed sequences are closely associated with the nuclear cage.

Samples of total DNA (100%) and DNA that resisted detachment by Eco RI (11% and 6%) were purified, completely recut with <u>Hae</u> III, subjected to electrophoresis in 2% agarose, blotted, hybridized with labelled probes (total RNA, a; poly A<sup>+</sup> RNA, b; rDNA, c) and autoradiographs prepared. The molecular weight markers are from PM2 DNA restricted with <u>Hae</u> III.

and 3 of the others are enriched in DNA resisting detachment (Fig. 7a, channels 3,4). Poly  $A^+$  RNA hybridizes to some of these in total DNA and they, too - as well as minor bands and the background smear - are enriched (Fig. 7b). These results clearly confirm that many transcribed sequences lie close to the cage.

## DISCUSSION

Three types of attachment of DNA to the cage have been proposed. One is structural, remaining throughout the cell-cycle (23), and fixes the DNA in loops of about 220 kbp so that sequences within them can be mapped. The other 2 are detected functionally and concern replicating (31) or transcribing (7,32) sequences. Because these functional attachments are likely to change constantly as replication and transcription proceed structural attachments may be best analyzed using mitotic cells where little replication or transcription occurs. In fact, similar results are obtained using nucleoids from cells synchronized in mitosis (cages from mitotic cells resemble, in many respects, chromosome scaffolds) or  $G_1$ (results not shown). Even with unsynchronized cells, attachment of replicating sequences is unlikely to affect the results desribed here, since few loops are replicating at any one time (31), though attachment of transcribing sequences could be responsible for some of our observations. Specificity of attachment

It is feasible that looping suggested by studies on isolated structures results from the artefactual association of long DNA strands with those structures during preparation. However, if this were the case attached sequences might vary from one structure to another within the population so that detachment of most nuclear DNA would lead to neither enrichment nor depletion of any given sequence in the attached or pelleted fraction. Our present results with various repeats (i.e. rDNA and some uncharacterized ones) - like our earlier ones with unique sequences (4,7) - provide no evidence for such random attachments: instead test sequences are either enriched or depleted in the cage-associated DNA.

## Attachment of rDNA

There are several hundred rDNA genes in HeLa cells, many of which are probably being transcribed at any time (33). As DNA is progressively detached by <u>Eco</u> RI from cages, rDNA is detached to a lesser degree (Table 1). Thus, when 5% remains there are about 30, 25 and 15% of the 5', middle and 3' fragments respectively consistent with attachment upstream of the 18S ribosomal RNA gene. On complete digestion (1.5% of the DNA remaining) the 3' 20 kbp <u>Eco</u> RI fragment is almost completely detached whilst 12% of the 5' ends and 7% of the middle fragments remain. These results point to attachments within the minority (i.e. <20%) of loci at sites within <u>both</u> 5.7 and 7.3 kbp fragments, though attachment at the 5' end is most probable. Clearly, these results are inconsistent with a simple model involving one attachment site <u>per</u> repeat. We are currently trying to delineate more precisely the "attachment sequences" within the rDNA locus.

This gradient of enrichment enables us to counter the criticism that DNA attachments are generated by precipitation of polymerase or nascent RNA and its associated protein on to the cage. Such artefactual attachment requires that the greatest enrichment is seen towards the 3' end of the locus where there will be a greater concentration of nascent RNP - providing that the polymerases are uniformly distributed (33). Identification of "attachment sequences"

A number of reports have described the complexity of DNA fragments (ranging in size from 140-3000 bp) attached to matrix or scaffold preparations following endonuclease digestion. However, analyses using solution hybridization have not revealed any sequence which is associated uniquely with these structures. Indeed, this approach may be severely limited. For example, an attachment sequence of 15 bp would represent only 2-5% of the DNA in residual fragments of 350-1000 bp, and so might not be detected. Clearly a precise description of the residual fragments used in such experiments is essential.

The limitations of solution hybridization prompted our analysis of the residual DNA by hybridization to Southern blots. In principle, any DNA that resists detachment by non-specific endonucleases (e.g. DNase or micrococcal nuclease) should be composed entirely of attached sequences. Confirmation that they are "attachment sequences" would be provided if - in turn - they hybridized to a greater extent with DNA that remained cage-associated following restriction endonuclease digestion. Indeed, we have shown this to be the case, though not to the predicted extent. DNA fragments which resist complete digestion with DNAse and micrococcal nuclease hybridize to characteristic repeats in bulk DNA - these same repeats are enriched when different quantities of DNA remain cage-associated. However, the most striking result emerging from this study is our general failure to detect any DNA fragments which are uniquely cage-associated. This forces us to examine our premises:

(i) Is the residual DNA (<0.05% of the total) free of contaminating DNA? Our residual DNA preparations do not contain a significant proportion of large DNA fragments which would dilute the concentration of attached sequences. However we do not know whether these residual DNA fragments are specifically or non-specifically cage-associated. Exhaustive digestion and washing procedures should minimize contamination especially as cages have little affinity for exogenous DNA (31). We cannot be confident about the complexity of the residual DNA: it clearly contains a significant proportion of fragments which hybridize to specific repeats, but may also contain sequences representative of the whole genome. Intriguingly, most mitochondrial DNA fragments turned out to be closely associated with nucleoids, probably reflecting the known association between mitochondria and cytoskeletal elements (34,35) also present in our preparations.

(ii) Are "attachment repeats" found solely at the base of the loops? If, like rDNA repeats, only some are attached at any time, we would expect to find the enrichments observed (i.e. only 5-10x, and not 50x, when 2% remains). This necessarily begs the question - why are only some attached?

(iii) Do "attachment sequences" cross-hybridize? Our fundamental premise is that homologies between the different sequences which are attached within nucleoids allow them to cross-hybridize. If this were so we might expect to see hybridization of these sequences to all size classes and only to specific bands when they occur in larger repetitive elements. The absence of such general hybridization (Figs 5d,6) suggests that such homologies do not exist under our standard hybridization conditions. Consequently, the experiments described in Fig. 5b,d were repeated with hybridization in 6x SSC and 10x Denhardt's solution at 4 or 20°C for 2 to 12 h followed by repeated washing in 6x SSC and 10x Denhardt's solution (Fig. 5c and unpublished). These much less stringent conditions yield stable hybrids between complementary fragments only 15 nucleotides long (29), but do not alter significantly the hybridization patterns of our residual probes. This must imply that if specific "attachment sequences" exist in our preparations their organization precludes the use of hybridization as an analytical technique. Centromeric DNA is perhaps the closest analogue to an "attachment repeat" of which we know the sequence. Interestingly, 2 conserved regions of only 14 and 11 bp are separated by unique sequences and as a result different centromeric fragments do not usually cross-hybridize (36).

Bearing these points in mind, it seems that "attachment sequences" will only be identified with difficulty using the approach described here. Therefore, we are currently attempting to devise functional assays for these elusive sequences.

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