
Mapping sequences in loops of nuclear DNA by their progressive detachment from the nuclear cage

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ABSTRACT

Nuclear DNA is organised into loops, probably by attachment to a supra-molecular structure. We describe a method which enables us to map the position of sequences within a loop relative to the point of attachment. Nuclear DNA is isolated unbroken by lysing HeLa cells in 2M NaCl to release structures which retain many of the morphological features of nuclei. Their DNA is supercoiled and so must remain unbroken and looped during lysis. Nucleoids are digested to various degrees with a restriction endonuclease and the cages - and any associated DNA - sedimented free from unattached DNA. The cage-associated DNA is purified and completely fragmented using the same restriction endonuclease. Equal weights of fragmented DNA are separated by gel electrophoresis, transferred to a filter and the relative amounts of the α , β and γ globin genes on the filter determined by hybridisation to the appropriate probes. The α genes, unlike the β and γ genes, resist detachment from the cage and so must lie close to the point of attachment to the cage. Our ability to map these genes implies that sequences cannot be attached at random to the cage; rather, specific sequences must be attached, so looping the DNA.

INTRODUCTION

It is now widely accepted that linear nuclear DNA is organised into loops, possibly by attachment to a sub-nuclear structure (1-4). A number of such structures - called variously the 'matrix', 'envelope', 'pore-complex', 'scaffold' and 'cage' - have been isolated by different authors from a wide range of cells, but they probably share the same basic constituents (5-10). Although sequences that anneal rapidly resist detachment from these structures on digestion with nucleases (11,12), there remains no evidence for any attachment of specific sequences to specific sites on the structure. We now describe a method which enables us to map the position of genes within a loop relative to the point of attachment. The fact that we can do this implies that sequences cannot be attached at random; rather, specific places along the DNA must be attached, so looping the DNA.

Our approach is to isolate the nuclear DNA of HeLa cells without breaking it. We do this by lysing living cells in a detergent and 2M NaCl to release

structures which retain many of the morphological features of nuclei. These nucleoids lack any organised membrane but contain naked DNA packaged within a flexible cage of RNA and protein. Their DNA is supercoiled so that the linear DNA must remain unbroken and looped during lysis. The cage, which is derived from both cytoskeletal and nuclear elements, protects the fragile DNA from breakage (1,9,13,14). We then partially digest the nucleoids to various degrees with a restriction endonuclease and sediment the cages - and any associated DNA - free from unattached DNA. [We have described the structure of these cages elsewhere (S.J. McCreedy et al., manuscript submitted for publication)]. Next the DNA associated with the cage is purified and completely fragmented by the same restriction endonuclease. Equal weights of fragmented DNA are separated by gel electrophoresis, transferred to a filter and the relative amounts of any sequence on the filter determined after hybridization to the appropriate probe. If sequences are associated at random with cages, detachment of DNA will lead neither to an enrichment nor a depletion of a particular sequence. On the other hand, if the association is specific a sequence close to the point of attachment of the DNA will resist detachment: those remote from the attachment site will be detached at a rate which depends on the number of restriction sites between them and the attachment site. In the experiments described here we establish the feasibility of the method of mapping the relative positions of the α , β and γ globin genes in the loops of HeLa cells using the restriction endonuclease, Eco RI.

MATERIALS AND METHODS

Cells

HeLa cells were grown in suspension (1) and synchronised by the nitrous oxide technique of Rao (15) as described by Warren and Cook (16). Cells in G1 were obtained 2 h after release of the nitrous oxide block (the mitotic index immediately on release of the block was routinely >90%). Cells (0.3×10^6 /ml) were labelled by growing them for 24 h in [methyl- ^3H]thymidine ($0.1 \mu\text{Ci/ml}$) prior to synchronisation or the preparation of nucleoids.

Chemicals

[methyl- ^3H]thymidine (58 Ci/mmol), [^{32}P]dATP and [^{32}P]dCTP (>2000 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, and restriction endonucleases from Boehringer or Bethesda Research Labs.

Preparation and digestion of nucleoids

HeLa nucleoids were isolated in bulk using 'step' gradients containing

1.95 M NaCl and stained, counted and manipulated as described (9).

Nucleoids ($1-2 \times 10^6$ /ml) were digested with endonucleases in 0.2 M NaCl, 2 mM MgCl₂ and 10 mM Tris (pH 8.0) for 10 min at 37°C. Different concentrations of Eco RI or Bam HI were used, ranging from $1-10^3$ units/ml. Control experiments showed that λ DNA was specifically cleaved by the restriction endonucleases under these conditions. Digestions were stopped by adding 500 mM EDTA, 10 mM Tris (pH 8.0) to give a final concentration of EDTA of 20 mM.

Filtration

The percentage of the DNA remaining attached to nucleoid cages after digestion was determined by filtration using glass-fibre discs: cages and any associated DNA are retained by the filter (S.J. McCready *et al.*, manuscript submitted for publication). Generally 30 μ l samples were applied to 2.5 cm GF/C discs (Whatman) and then 30 ml 2 M NaCl 0.1% tetrasodium pyrophosphate, 1 mM Tris (pH 8.0) was sucked through the discs at a flow rate of 0.5 ml/sec. Any radioactivity associated with the cage was then measured using a Packard Tri-Carb liquid scintillation spectrometer after immersing the disc in 3 ml Unisolve I (Koch-Light Ltd.).

The following control experiments showed that DNA associated with the nucleoid cages remained on the disc after filtration whereas free DNA was removed. Nucleoids were prepared from cells grown in [³H]thymidine or [³H]leucine for 24 h and applied to the discs: after filtration >90% of the applied label remained associated with the discs. When a mixture of unlabelled nucleoids and pure HeLa [³H]DNA was applied to discs, >90% of the label was lost from the disc on filtration. This was true, whether or not the DNA had been cut with Eco RI. [DNA of very high molecular weight released during partial digestion of nucleoids might be expected to be removed from the discs less efficiently than the pure DNA prepared by conventional procedures used in these controls. Unfortunately, as we cannot isolate pure DNA of equivalent molecular weight, we cannot determine this. However, an underestimate of the degree of digestion does not affect the principal qualitative conclusions of this study.]

Purification and redigestion of DNA associated with cages

Digested cages were freed of detached DNA fragments by sedimentation (35,000 rpm; 15 min; Beckman SW41 rotor) through 1 ml 5% sucrose containing 2 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA. DNA was purified from the pellet essentially as described by Gross-Bellard *et al.* (17). This method involves digestion with proteinase K in sodium dodecyl sulphate, phenol and

then ether extraction, dialysis, digestion with ribonuclease, phenol and then ether extraction, dialysis and precipitation with ethanol. The precipitate was dissolved in 10 mM Tris (pH 8.0) and the DNA concentration determined from the optical density and radioactive content.

Equal weights of DNA (~5 µg) were completely redigested for 4 h at 37°C with Eco RI in 100 mM tris-HCl (pH 7.5), 50 mM NaCl and 10 mM MgCl₂ or Bam HI in 20 mM Tris (pH 8.0), 7 mM MgCl₂ and 100 mM NaCl. The reaction was stopped by the addition of NaCl to 0.5 M, the DNA precipitated with ethanol and redissolved in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA (pH 8.3) prior to electrophoresis.

Electrophoresis, "blotting" and hybridization

Samples were subjected to electrophoresis in 0.8% agarose and then transferred to nitrocellulose filters (18). λ DNA (Boehringer), restricted with Eco RI or Hind III, served as marker DNA (19,20). [³²P]-labelled probes were prepared by nick-translating plasmid DNA using [³²P]dCTP and [³²P]dATP (21). The plasmids used were JW 101, 102 and 151, which contained α, β and γ-globin cDNA respectively (22). The DNA on the filters was hybridized to the [³²P]-probes (21), the filters washed using stringent conditions (23) and the autoradiographs prepared using 'preflushed' (24) Fuji-RX X-ray film and Ilford Fast Tungstate intensifying screens. Developed autoradiographs were scanned using a Joyce-Loebl microdensitometer and peak heights measured.

For these experiments, it is important that (i) equal weights of DNA be applied to the gel, (ii) samples should be completely restricted during the second digestion (iii) "blotting", hybridization and autoradiography be uniform across the gel or filter and (iv) the band intensities in the autoradiographs should reflect the concentration of specific sequences in the gel. The first 2 points were checked after electrophoresis by analysis of photographs taken under UV-illumination of the gels stained with ethidium. The third point was monitored in one (or both) of two ways. Samples of pure HeLa DNA, completely digested with the restriction endonuclease were included as flanking markers on all gels: after "blotting" and hybridization, the intensities of the bands in the autoradiographs of the flanking markers were compared. Alternatively 0.1 ng of PM2 DNA (Boehringer) cut once with Hpa II (25) served as a suitable internal marker for inclusion in all the DNA samples applied to the gel: subsequently 10 ng of "nick-translated" PM2 DNA (containing ~2 x 10⁵ cpm of [³²P]) was included in the hybridization mixture. This procedure yields a PM2 band in the auto-

radiograph of roughly equal intensity to the globin bands. The linearity of response (i.e. iv) was determined by correlating band intensities given by different amounts of DNA applied to the gel in channels flanking the experimental samples. A number of autoradiographs were prepared from each filter and only those giving a linear response over a fivefold range of DNA concentration (i.e. 2-10 μg) were used. Examples are given in Figs 1 and 5. Preliminary experiments showed that band-height and band-area of the microdensitometer traces gave equivalent results, and these correlated with the radioactive content in the filter determined by liquid scintillation counting.

We stress that we do not yet attach too much weight to the quantitative aspect of this work - an aspect that should yield significant mapping data - until we have accumulated greater experience on the influence of background labelling, the linearity of response over a wider range and the assessment of the degree of detachment of DNA. An indication of the variation that we find is given in the figures. Nevertheless, we should add that the general qualitative trends described are quite clear and can be observed even in autoradiographs with very high backgrounds.

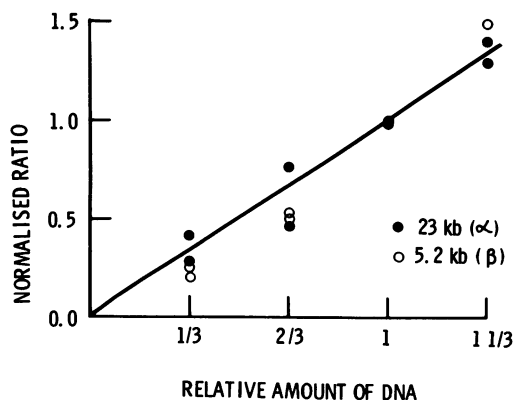


Fig. 1. The linearity of response.

Total HeLa nucleoid DNA was purified, completely fragmented with Eco RI and different amounts subjected to electrophoresis. After blotting, hybridization with α and β probes, autoradiography (the autoradiograph is presented in Fig. 5) and densitometry, peak heights of the 23kb (α) and 5.8 kb (β) bands were measured. Peak heights (\bullet , α fragment; \circ , β fragment) were normalised (relative to the height in one channel) and plotted against the DNA concentration (expressed as a multiple of the concentration in the one channel).

RESULTS

We have shown elsewhere that > 95% of the loops can be cut at least once by Eco RI (S.J. McCready *et al.*, manuscript submitted for publication). [The loops are so large (i.e. 220 kb) that they would be expected to contain many (i.e. > 50) potential cutting sites.] We have also shown that replicating DNA is closely associated with nucleoid cages so that all sequences must briefly become attached to the cage as they are replicated. Since these attached replicating sequences might obscure other specific associations, we initially used non-replicating cells synchronised in G1. Subsequently we obtained qualitatively similar results using unsynchronised cells. The advantage of using synchronised cells proves slight because so little DNA in a population of unsynchronised cells is replicating and so attached at any time (see Discussion).

We first mapped the position of the two α -globin genes using Eco RI. When human DNA is completely digested with Eco RI, both α genes are contained in one fragment of 23kb (26). This fragment can be seen as a band in autoradiographs following hybridization of [³²P] α -globin cDNA to Southern "blots". In Fig. 2, the band given by DNA purified from undigested nucleoids is very faint (channels 1 and 7). We use its intensity as a reference. When nucleoids are digested with Eco RI - to leave only 86% of the DNA remaining attached to the cage - and then their DNA isolated and completely fragmented

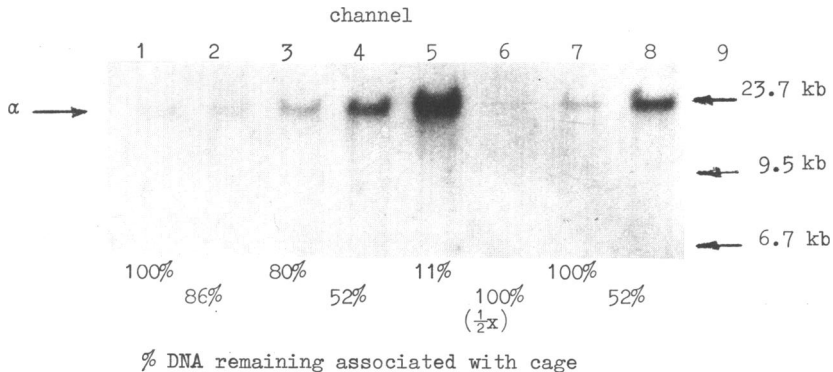


Fig. 2. The α -genes are enriched by detaching DNA with Eco RI. Nucleoids were digested with Eco RI and the percentage of DNA remaining associated with cages determined by filtration. After purifying the DNA, redigestion, electrophoresis, blotting and hybridization with α -cDNA probe, an autoradiograph was prepared and photographed. Equal amounts of DNA were applied to all channels except 6 which contained half the amount. The position of flanking size markers (λ /Hind III) is indicated on the right.

with Eco RI, the band obtained subsequently is more intense (Fig. 2, channel 2). As DNA is progressively detached during the initial partial digestion of nucleoids, the band obtained after the second complete digestion becomes progressively intenser (Fig. 2 channels 2-5). When all but 20% of the DNA

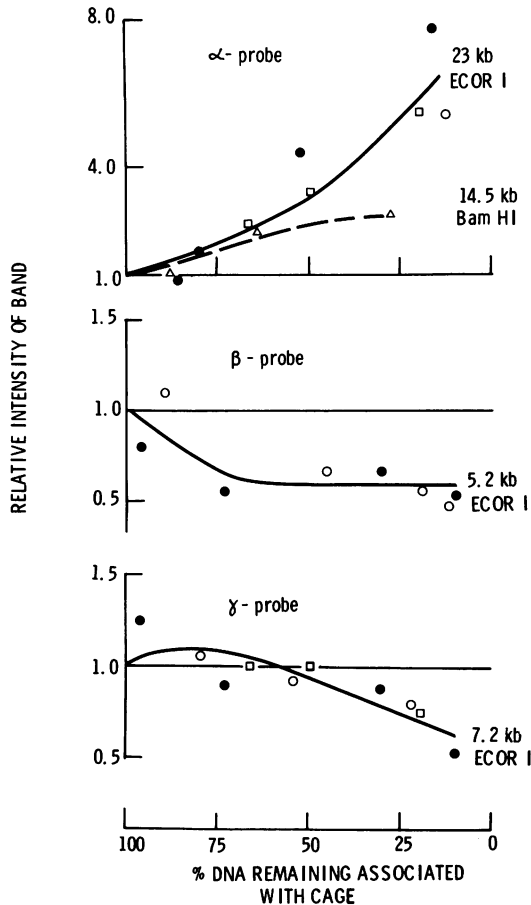


Fig. 3. The relative concentration of α , β and γ genes in DNA remaining associated with cages.

Nucleoids were digested either with Eco RI or Bam HI and the percentage of DNA remaining associated with cages determined. After purifying the DNA, fragmenting again with the same endonuclease, electrophoresis, blotting and hybridization with the appropriate probes, autoradiographs were prepared and analyzed by microdensitometry. The intensities of the bands are expressed as a ratio relative to those given by the DNA of undigested cages. Different symbols refer to different experiments with Eco RI, except for the one with Bam HI (Δ).

is removed it becomes about 5x darker (Fig. 3). Clearly, the α -globin sequences constitute a greater proportion of the remaining DNA: they resist detachment and so few, if any, restriction sites can lie between them and the cage.

In contrast to α -globin, the β and γ genes are detached easily from the cages (Figs 3-5). For example, the β and γ cDNA probes hybridize strongly to Eco RI fragments of 5.2 and 7.2 kb respectively (27). [The β and γ genes lie next to each other, on a different chromosome from that carrying α]. The relative intensity of the 5.2 kb β band decreases as DNA is detached, whereas that of the 7.2 kb band increases slightly and then decreases (Fig. 3). The latter behaviour results from an initial detachment of DNA which leads to enrichment of the γ -genes, but then further digestion detaches them.

The enrichment of the α but depletion of the β and γ sequences can be highlighted by hybridizing a mixture of probes (i.e. either $\alpha + \beta$ or $\alpha + \gamma$) to the filters (Figs. 4,5). Whereas the α -band becomes darker on DNA detachment, the β and γ bands become relatively weaker. This observation rules out the trivial objection that we are not applying equal weights of DNA to each channel in the gel, for we see both enrichment and depletion of sequences within one channel. These figures also illustrate the uniformity

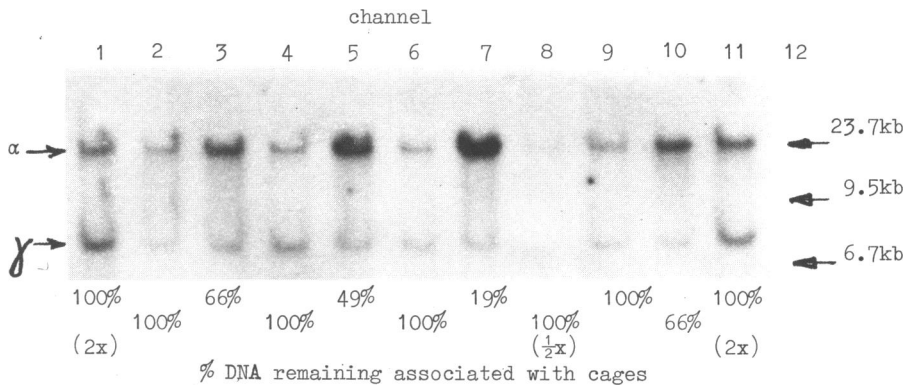


Fig. 4. Differential enrichment of the α and γ -genes

Nucleoids were digested with Eco RI and the percentage of DNA remaining cage-associated determined. After purifying the DNA, redigestion, electrophoresis, blotting and hybridization with a mixture of α - and γ -cDNA probes, an autoradiograph was prepared and photographed. Equal amounts of DNA were applied to all channels except 1, 11 and 8 which contained twice and half the amounts respectively. The position of flanking size markers (λ /Hind III) is indicated in channel 12.

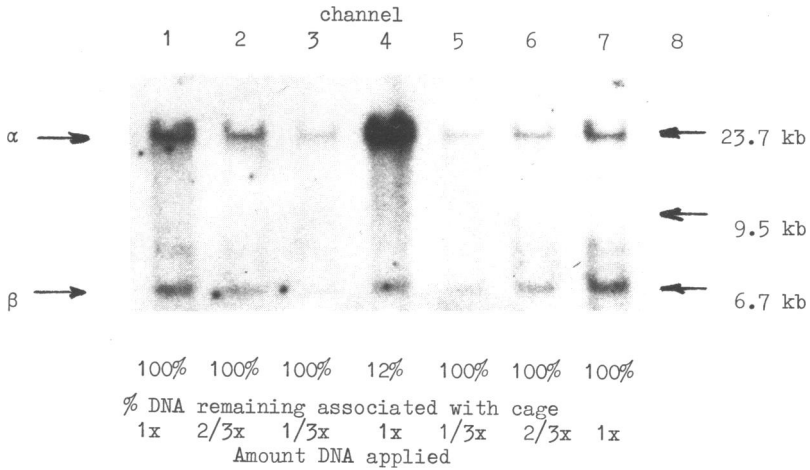


Fig. 5. Differential enrichment of α and β genes.

Nucleoids were digested with Eco RI and the percentage of DNA remaining associated with cages determined. After purifying the DNA, redigestion, electrophoresis, blotting and hybridization with a mixture of α - and β -probes, an autoradiograph was prepared and photographed. Different amounts of DNA (expressed as multiples) were applied to the various channels as indicated. The position of flanking size markers (λ /Hind III) is indicated in channel 8.

of transfer and hybridization across the filter that can be achieved.

We are currently mapping more precisely the attachment points of these genes using different probes and different restriction enzymes. An example of the mapping of the α genes using Bam HI instead of Eco RI is illustrated in Fig. 3. The α genes are contained in a fragment of 14.5 kb when DNA is cut by Bam HI (26). The intensity given by this fragment increases slightly as DNA is detached, but this is not nearly as much as the increase found with the 23 kb Eco RI fragment. These results are consistent with an attachment point lying within the Eco RI fragment, but outside the smaller Bam HI fragment.

DISCUSSION

During the last few years methods have been established for determining the sequences of bases within the mammalian genome, but as yet we know little about how these sequences are organised in three dimensions within the nucleus. Our result that the α globin genes are enriched fivefold by

detaching all but a fifth of the DNA indicates that sequences cannot be associated at random with cages. Rather, the globin sequences must be organised in space in a highly specific manner by attachment to the cage. We need make no assumptions as to how DNA is attached: the association is defined operationally in the sense that more or less DNA remains with the cage.

There must be 2 kinds of attachments that are stable in the detergent and 2 M NaCl used to isolate nucleoids. The first is the one that we have been concerned with here - specific sequences scattered along the linear DNA are attached, looping the DNA. Since no change can be detected in the average size of the loop throughout the cell cycle, these associations must be very stable and maintained even through mitosis (16). However, it remains to be shown whether or not genes retain their specific relation to these attachment points as cells differentiate. The second kind of attachment has been described elsewhere (S.J. McCreedy et al., manuscript submitted for publication) : newly-replicated DNA resists detachment so that replicating forks must also be attached. When sequences are replicated, they briefly become attached as they pass through a fixed replication complex at the cage. Since so few globin sequences are being replicated at any time in an unsynchronised population, this association of replicating sequences does not obscure the additional specific attachments. [We calculate that in an unsynchronised population $< 3\%$ of all loops are replicating at any time and only a fraction of each loop is attached]. Our results cannot be explained by differential rates of replication - and hence attachment - of the various globin genes since we obtain similar results using non-replicating cells.

Our approach permits us to map the position of any gene for which we have a probe relative to its point of attachment to the nuclear cage. In principle we can map the relative positions of genes in one of two ways. The first is the coarse approach described in Fig. 3 - we compare band intensities in adjacent channels loaded with equal amounts of DNA purified from cages from which different amounts of DNA have been detached. A finer map can be constructed using mixtures of probes and by comparing the relative intensities of bands in any one channel (see Figs 4 and 5). Eventually we hope to express map distances in terms of numbers of restriction sites between sequences or between a sequence and the attachment point.

What are the likely short-comings of the method? One is that restriction sites may be protected by the RNA in nucleoids, especially if the site lies

in an actively-transcribed gene. Another limits the quantitative power of the method. A fraction of detached sequences associate non-specifically with the cages. At low levels of digestion, this fraction will be insignificant in proportion to the DNA that is specifically tied to the cage, but as specific sequences are detached, the non-specific fraction becomes more significant. It is probably this non-specific association that prevents us from seeing the complete detachment of the β and γ genes by high levels of digestion. Instead these genes become depleted to a constant level which is about half that of undigested controls. Because of this restriction on the method, we have not yet attempted to interpret our data quantitatively to give a more precise map of the genes.

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