Attachment of DNA to the nucleoskeleton of HeLa cells examined using physiological conditions

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ABSTRACT

Although it is widely believed that eukaryotic DNA is looped by attachment to a nucleoskeleton, there is controversy about its composition and which sequences are attached to it. As most nuclear derivatives are isolated using unphysiological conditions, the criticism that attachments seen *in vitro* are generated artifactually has been difficult to rebut. Therefore we have re-investigated attachments of chromatin to the skeleton using physiological conditions. HeLa cells are encapsulated in agarose microbeads and lysed using Triton in a 'physiological' buffer. Then, most chromatin can be electroeluted after treatment with a restriction enzyme to leave some at the base of the loops still attached. Analysis of the size and amounts of these residual fragments indicates that the loops are 80 – 90kbp long. The residual fragments are stably attached, with about 1kbp of each fragment protected from nuclease attack. This is very much longer than a typical protein-binding site of 10 – 20bp.

INTRODUCTION

It is widely believed that eukaryotic DNA is looped within the interphase nucleus by attachment to a nucleoskeleton (1) and there is some evidence that the loops are important units of function (2,3). However, there is little agreement as to the composition of the nucleoskeleton or the structure of the points of attachment (4). Much of the controversy stems from the use of unphysiological isolation conditions, with different procedures yielding different structures. Nuclei are often isolated in buffers containing about 1/10 the physiological salt concentration (5) because chromatin aggregates in isotonic salt concentrations (6). However, we have recently shown that this apparently gentle procedure generates an equal number of new attachments (7). Such isolated nuclei may then be extracted with detergents or high concentrations of salt to yield nuclear 'matrices' and 'scaffolds' (1,5,8), treatments which further distort attachments (7). As a result, structures isolated in different ways can be very different from each other.

One attractive way of assessing how close a particular isolate might be to a structure found *in vivo* is to see whether **specific** sequences of DNA are attached to it. Artifactual binding of DNA occurring *in vitro* is assumed to be sequence independent, with specific attachments being more likely to reflect pre-existing structures (9). Early studies using such a 'detachment mapping' technique showed that replicating and transcribing sequences, and enhancers, were associated with the sub-structure (for reviews see refs 2,3), but subsequent studies have obtained conflicting results (8,10–12; reviewed in ref. 4). For example, the most detailed mapping has been carried out in scaffolds, but the attached sequences are generally not transcribed (8). These different results may be ascribed to an uncovering of different sub-sets of attachments or to a specific aggregation during isolation of the various structures. Lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase provide an extreme example of the kind of artifact that might occur; both enzymes are unlikely to play a role in the nucleus but both can be induced to form **specific** complexes *in vitro* with DNA and the DNA polymerase-α-primase complex (13).

Because of problems like these, we have developed an isolation procedure that uses conditions as close as conveniently possible to the physiological. Then, preservation of function can be used to assess—albeit indirectly—preservation of structure. Cells are encapsulated in agarose microbeads (14) and as protein complexes as large as 1.5×10^8 daltons can diffuse through the agarose, the encapsulated cells are completely accessible to molecular probes. Embedded within the microbead they are protected from shear and can be transferred without aggregation from one buffer to another simply by pelletting. Then cells can be lysed with Triton in a 'physiological' buffer (pH 7.4) which 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 (15). Whilst the precise ionic constitution *in vivo* remains unknown, we cannot be certain that the resulting *in vitro* preparation is free of artifact, but we do know that it initially contains **intact** DNA and essentially **all** the replicative and transcriptional activities of the living cell (15); if artifacts occur, they cannot interfere with vital functions.

In view of the continuing controversy and the potential importance of loops, we are currently re-investigating DNA attachments in such structures. HeLa cells are labelled with [^{3}H] thymidine, encapsulated and lysed. Beads are then incubated with different amounts of a restriction enzyme and subjected to electrophoresis in the 'physiological' buffer; detached chromatin migrates out of beads to leave the base of the loops still attached and function relatively unaffected (15). We have shown elsewhere

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that traditional procedures all dramatically affect the number of attachments (7). We now examine how the various steps in the procedure affect the number of residual fragments and go on to measure the size of the attached region.

MATERIALS AND METHODS

Cells:- HeLa cells were grown as suspension cultures in minimal essential medium plus 5% newborn calf serum. In most experiments cells were grown for 18–24h in [methyl-^{3}H] thymidine (0.05μCi/ml; ~60 Ci/mmol) to label uniformly their DNA. This enabled corrections to be made subsequently for any slight variations in cell numbers.

Lysis:- Cells were encapsulated (2.5×10^6 cells/ml unless stated otherwise) in agarose microbeads, the beads filtered through nylon to remove any large ones, washed in a 'physiological' buffer (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, pH 7.4) and lysed by washing in 3 changes (15 min. each) 10vols. 0.5% Triton X100 in the buffer (15).

Determination of loop size:- A typical procedure is given using the 'physiological' buffer from lysis to final sample analysis. Samples were kept at 4°C except during nuclease digestion. Following lysis, beads were washed (3×5 min), resuspended in an equal volume, incubated with *Hae*III (30 min. at 32°C), split, and half subjected to electrophoresis in the buffer to remove detached chromatin (1v/cm for 15h or 3v/cm for 5h; buffer recirculated to prevent pH drift). In some cases, chromatin was removed by field inversion electrophoresis (0.3s forward and 0.1s backward, linearly ramped to 9s forward and 3s backward over 14h at 2v/cm; ref. 16). Beads were recovered, protein removed (0.2% SDS plus 50μg/ml proteinase K; 37°C; 5h.), applied to a 0.8% agarose gel and their DNA sized electrophoretically by applying equal numbers of beads to each track (7). In some cases RNA in the gel was removed by treatment with 0.5% N-laurylsarcosine (sarkosyl) and RNAase (2μg/ml; 1h; 37°C). After ethidium staining and photography, gel tracks were sliced and slices (0.25cm) dissolved in 0.5% SDS, 1M HCl (90°C; 5min.), ^{3}H counted and weight average molecular weights determined.

Number average molecular weights were then calculated from the weight averages (17) and finally loops sizes calculated (18). For example, in Fig. 1B lane 4, the ^{3}H in each slice was measured and the weight fraction, F(L), plotted against fragment size, a. The weight average molecular weight (ie 6.6kbp at F(L) = 0.5) of the distribution was obtained from the experimental results. Six theoretical curves were obtained by substituting values of 1/P of 3, 3.2, 3.4, 3.6, 3.8 and 4 respectively into $F(L) = (1+Pa)e^{-Pa}$, where 1/P is equivalent to the number average molecular weight, and then evaluating F(L) for different values of a. 1/P = 3.8 gives the best fit between the experimental data and the theoretical expression (ie the number average molecular weight is 3.8kbp).

Enzyme digestions:- Restriction endonuclease (Amersham) and exonuclease III (Boehringer) digestions were performed in the unmodified 'physiological' buffer (see above) at 32°C. The buffer was modified for other nucleases as follows: pancreatic DNase (Amersham, 60units/μl), none; micrococcal nuclease (Worthington, 45units/μl), 1mM $CaCl_2$; Bal31 (Boehringer, 2.5units/μl), 1mM $CaCl_2$, 200mM NaCl; Mung bean nuclease (Sigma, 25units/μl), 0.1mM $ZnSO_4$ and pH reduced to 6.5 with HCl. When necessary, histones were stripped from residual chromatin fragments prior to nuclease digestion by mixing beads (15min, 4°C) with physiological buffer supplemented with NaCl to give a final monovalent ion concentration of 2M.

Isolation of DNA from beads:- DNA in residual chromatin fragments was purified by washing beads in 10mM Tris-HCl (pH 7.4), 1mM EDTA and 50mM NaCl, and incubation in 0.2% SDS, 50 μg/ml proteinase K (5h, 37°C). To recover detached fragments, DNA was electroeluted from beads (1ml beads in 3ml buffer in a dialysis bag), and the supernatant extracted with phenol/chloroform and precipitated with ethanol (19).

DNA was purified from detached chromatin fragments as follows. Beads containing digested material were subjected to electrophoresis in a dialysis bag (3v/cm for 5h; buffer recirculated to prevent pH drift). After recovering the contents of the bag, beads were pelleted and DNA purified from the supernatant by treatment with SDS and proteinase K as above.

RESULTS

Estimation of loop size

Loop size is determined using the approach of Igo-Kemenes and Zachau (18). Chromatin is cut with a restriction enzyme and any fragments detached from a sub-structure are removed electrophoretically. Then loop size is calculated from the percentage of chromatin (ie ^{3}H) remaining in beads and the size of attached fragments (determined by gel electrophoresis after purification). We illustrate the approach using two restriction enzymes that cut chromatin into differently-sized pieces (ie *Eco*RI and *Hae*III) and two different electrophoretic fields (ie conventional or field-inversion fields) to remove and size the resulting fragments. Accurate estimation of loop size requires that (i) each loop is cut so that chromatin can be detached, (ii) all detached fragments are removed and (iii) residual fragments are sized accurately.

Treatment with increasing quantities of either enzyme followed by gel electrophoresis progressively removes more chromatin (ie ^{3}H) from beads (Fig. 1A). As expected, *Hae*III treatment leads to removal of more chromatin than *Eco*RI, which cuts less frequently.

After removing detached fragments, DNA remaining in beads was purified and sized on a second (conventional) gel (Fig 1B, lanes 1–4). This DNA is assumed to be retained because it is at the base of the loop and attached to the skeleton. Unattached DNA could also be retained because it was too large to escape through the pores in the bead: this possibility is eliminated later. After treatment with high levels of *Eco*RI, 21% of DNA remained in beads and nearly all of this was >5kbp, with most being too large to be resolved (Fig. 1B, lane 2). Therefore its weight average molecular weight could not be determined accurately. However, treatment with *Hae*III cuts chromatin into smaller fragments that contain DNA that is well resolved (Fig. 1B, lanes 3,4). With high levels of *Hae*III (lane 4), a fraction (ie 2.05% ±SD=0.25; n=10) remains at the top of the gel. This results from a satellite devoid of *Hae*III sites which is also found when naked DNA is digested (not shown). As analysis depends on random scattering of restriction sites, this satellite is excluded from subsequent analysis, but it does provide an internal control that loadings are correct. This satellite remains in beads as

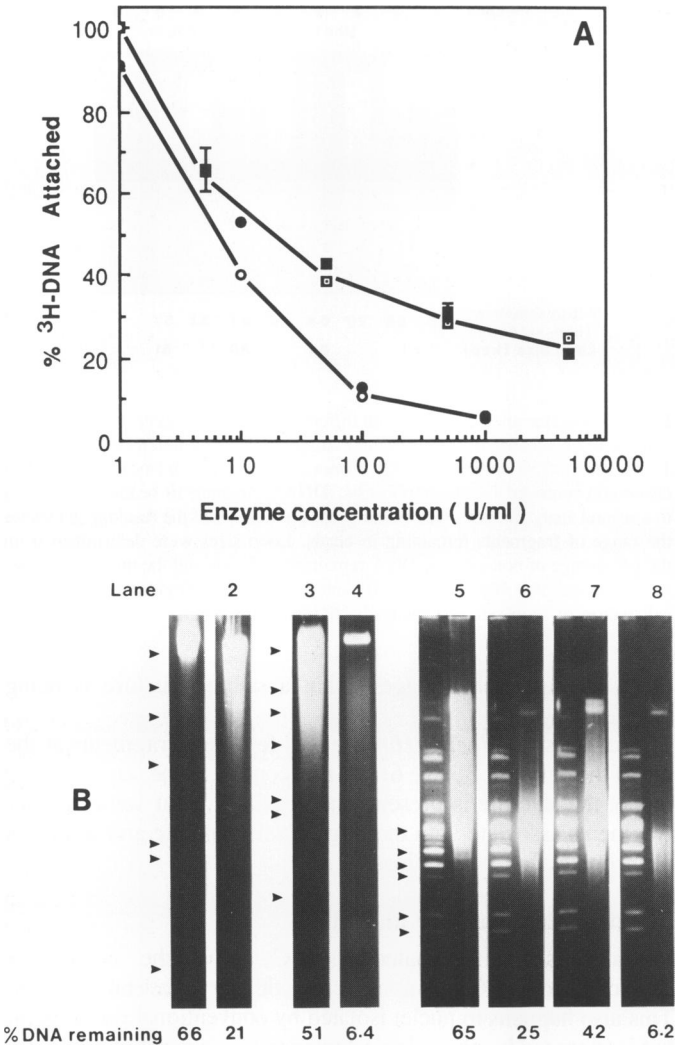


Fig. 1. Estimation of loop size by nuclease digestion. [^{3}H] labelled cells were encapsulated, lysed, incubated (32°C; 30min.) with different concentrations of an endonuclease and unattached chromatin fragments removed electrophoretically. (A). Graph showing the percentage of DNA remaining in beads after digestion with different amounts of *Eco*RI (squares) or *Hae*III (circles) and then removal of detached chromatin by conventional (closed symbols) or field-inversion (open symbols) gel electrophoresis. (B). Photographs of gels to illustrate the range of DNA fragments remaining in beads after digestion with *Eco*RI (lanes 1,2,5 and 6) or *Hae*III (lanes 3,4, 7 and 8). Chromatin was both removed and attached DNA analyzed by conventional (lanes 1–4) or field-inversion (lanes 5–8) electrophoresis. Arrowheads: λ/*Hind*III markers. Additional markers (0.6–125kbp) were run in alternate lanes for field inversion gels.

chromatin but can be removed by field inversion electrophoresis after treatment with SDS.

Loop size is determined using Fig. 1B, lane 4 as follows. The weight average molecular weight of the DNA in the gel (ie 6.4kbp) is determined by slicing the gel and counting the ^{3}H in each slice. The equivalent number average molecular weight (ie 3.8kbp) can then be calculated by standard methods (17; see Materials and methods). 6.4% chromatin remained in beads and, of this, 4.35% was non-satellite and had a number average molecular weight of 3.8kbp; then the average loop size is $100/4.35 \times 3.8 = 87$kbp. The results of 24 different experiments (involving a range of different loadings and degrees of digestion) gave an average loop size of 87kbp (SD = ±8; range 68–118kbp).

*Hae*III does not cut within nucleosomes in these experiments. This is demonstrated by comparing fragment sizes that result from completely cutting total chromatin and pure DNA; then their ratio (ie 1700bp:730bp) exactly equals the ratio of the lengths of the protected nucleosomal core and accessible spacer (ie 140bp:60bp). Furthermore clear nucleosomal repeats can be seen with total chromatin (but not with pure DNA) on analytical gels (results not shown).

Electrophoresis removes detached fragments efficiently

Although chromatin fragments containing DNA of 150kbp can electroelute through agarose (20), a fraction of the fragments might be so **entangled** that they cannot escape from the nucleus. However, detached chromatin was removed as efficiently by field inversion electrophoresis (Fig. 1A)—a procedure which should untangle such complexes. Detached chromatin fragments might also fail to electroelute because they **aggregate** into large complexes. This seems unlikely as the extent of removal (and measured loop size) is exactly the same in the Mg^{2+}-free buffer at a different pH (ie pH 8.0) used by Jackson and Cook (14; not shown), where aggregation and the electrophoretic mobilities of the resulting complexes would be expected to be different. Therefore we have no reason to believe that detached chromatin is not removed efficiently by electrophoresis, but if aggregation occurs, loop size is underestimated.

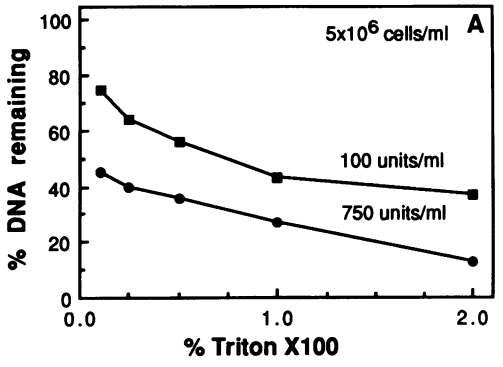
Effect of different detergents during lysis

Chromatin fragments might also fail to electroelute because membranes were inefficiently permeabilised. Indeed, in preliminary experiments we discovered this to be a real problem; the measured loop size appeared very sensitive to the efficiency of lysis. Presumably detached chromatin, which was nevertheless cut efficiently (not shown), could not escape. For example, the Triton concentration during lysis affects measured retentions—and hence loop size—over a fourfold range (Fig. 2A). [Note that in such experiments it is important to maintain a constant cell concentration; the higher the cell concentration the more detergent required for efficient lysis.] Consecutive washes with non-ionic detergents eventually led to the same retentions (and loop sizes), the stronger detergents needing fewer washes (Fig. 2B). This suggests that eventually all membranes are disrupted and that a stable structure remains. Therefore we generally use the minimum exposure giving this limit (see Materials and methods). Other agents that lyse cells efficiently without extensively disrupting membranes (eg mellitin or antibody plus complement; Fig. 2C) allowed the nuclease to enter and cut the chromatin (15) but did not allow quantitative electrophoretic removal of detached fragments.

Although these detergents give similar results when used carefully, they all act in roughly similar ways and might destroy pre-existing attachments; this would lead to an over-estimation of loop size.

Effects of different restriction enzymes

*Eco*RI fragments too large to be resolved in conventional gels can be resolved by field-inversion electrophoresis (Fig. 1B, lanes 5 and 6). Unfortunately, these *Eco*RI fragments are not separated by our field inversion gels as effectively as are *Hae*III fragments in conventional gels (cf lanes 2 and 4 with 6 and 8) so estimates of molecular weights (and so loop size) are less accurate. There is an additional theoretical difficulty in using an enzyme like *Eco*RI that cannot cut chromatin into small fragments; we require



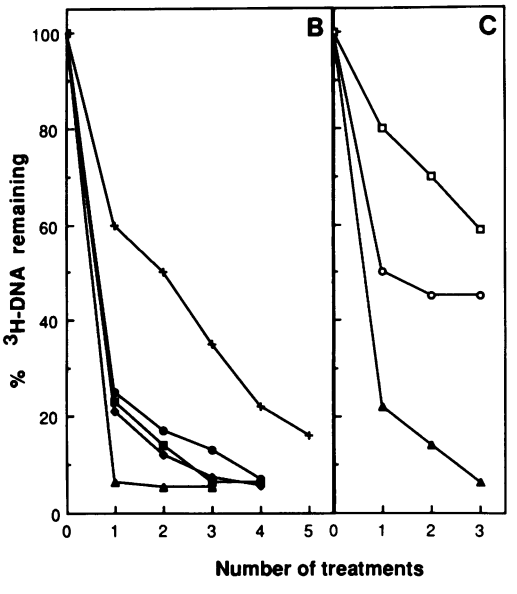


Fig. 2. Efficiency of lysis. [^{3}H] labelled cells were encapsulated (5 or 2.5×10^6 cells/ml in A or B and C respectively), lysed in various ways, washed, treated with *Hae*III (100 or 750 units/ml; 32°C; 30min.) and the ^{3}H remaining in beads after electrophoresis measured. (A). Lysis by a single incubation in the buffer plus Triton X100 at the concentration indicated. (B). Lysis by up to 5 consecutive treatments (0°C; 15min.) in 10 vol. buffer plus (■) 0.5% Triton X100, (♦) NP40, (●) Tween 20, (+) Brij 35 and (▲) Lubrol PX. (C). As (B) with up to three treatments with (△) 100μg/ml lysolecithin for 5min. at 0°C, (□) 25 μg/ml mellitin for 5min. at 0°C or (○) complement for 10min. at 20°C (15).

that each loop be cut at least once. We have shown elsewhere that about 20% of loops are of the size of, or smaller than, these *Eco*RI fragments (7) so that some loops are unlikely to be cut. Then the apparent loop size should be larger than that determined using small fragments (ie using high concentrations of *Hae*III); this proves to be the case at digestion levels that leave 20–50% DNA attached (ie loop size of 116.3 ± 13kbp; n = 5; results not shown). Therefore we routinely use high concentrations of *Hae*III and conventional gel electrophoresis to remove all but 5–20% DNA; then estimates of loop size fall in the range of 75–95kbp. Estimates using *Hae*III and field inversion give a larger scatter of loop size, due almost entirely to the inaccuracies in sizing which result from the lower resolution of the gels (results not shown).

As expected, other enzymes that cut as frequently as *Hae*III (ie *Mbo*I, *Hin*fI) each gave characteristic digestion profiles, partial digestion products and limit retentions but the same loop size

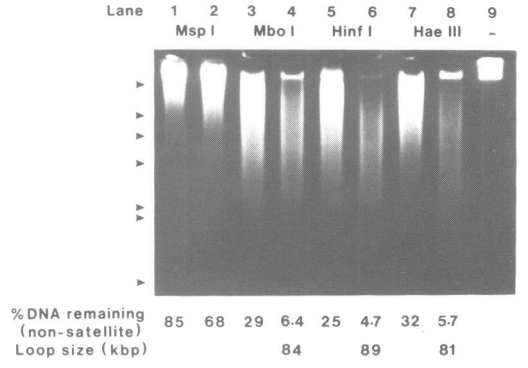


Fig. 3. Loop size after digestion with different restriction enzymes. [^{3}H] labelled cells were encapsulated, lysed, treated with different restriction enzymes (lanes 1–8; 100 or 750 units/ml; 32°C; 30min.; lane 9, no enzyme) and detached chromatin removed by electrophoresis. DNA remaining in beads was applied to a second analytical gel. After staining, the photograph of the resulting gel shows the range of fragments remaining in beads. Loop sizes were determined from the percentage of non-satellite DNA remaining in beads and the number average molecular weights. *Msp*I cut too inefficiently in the 'physiological' buffer to allow calculation of loop size. Arrowheads: λ/*Hind*III markers.

(Fig. 3). Again this suggests that a stable structure is being examined.

In contrast to *Hae*III, *Hin*fI leaves few long fragments at the top of the gel (Fig. 3, lane 6). As the loop size is the same whether or not the satellite is present, the possibility that some smaller fragments associate with the large *Hae*III satellite and so do not electroelute can be eliminated.

Effects of temperature and pH

When cells are incubated 3–5°C above the normal, a characteristic set of proteins associate with karyoskeletal elements. This also happens to nuclei isolated by conventional procedures, but is triggered by physiological temperatures; isolation sensitizes nuclei (21–24). Therefore we routinely digest at 32°C—conditions that have been shown not to induce this 'heat-shock' response (15,22), but also obtain the same results at 37°C (not shown).

Quite small changes in pH in the physiological range dramatically affect the aggregation of chromatin (25). However, we obtained the same loop size after carrying out the whole experiment except for the restriction digest at pH 8.0 in a Mg^{2+}-free buffer (see above) and, in addition, the residual chromatin fragments remain stably attached in the 'physiological' buffer between pH 6.5–8.5 (results not shown). If large aggregates were formed in our buffer, they should disaggregate and be lost from beads, somewhere within this range.

Stability of bound DNA fragments

Nucleosomes are prone to redistribute or 'slide' along DNA when chromatin is isolated, especially when the tonicity is changed (26–29) and such a redistribution might alter attachments. However, the experiment illustrated in Fig. 4 shows that our preparation is not prone to such nucleosomal rearrangement. Many *Eco*RI sites are inevitably covered by nucleosomes and so remain uncut, leading to partial digestion products. Such products from the A^γ and G^γ globin loci can be visualised after electrophoresis, blotting and hybridisation with an appropriate probe. A stable pattern of such products remains irrespective of the length of incubation (not shown) or amount of *Eco*RI used

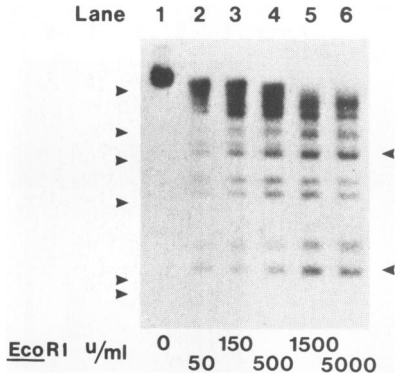


Fig. 4. Chromatin stability during prolonged digestion with *Eco*RI. Encapsulated cells were lysed using the standard conditions and incubated with *Eco*RI (0–5000 units/ml; 60 min.; 32°C). DNA from 10^6 cells was purified, subjected to electrophoresis on a 0.8% agarose gel, blotted on to a filter and the filter hybridised under stringent conditions with a probe complementary to two globin fragments of 2633bp (A^γ) and 6981bp (G^γ) in a complete digest of human DNA (9,19). Arrowheads: λ/*Hind*III markers (left) and globin fragments of 2633 and 6981bp (right).

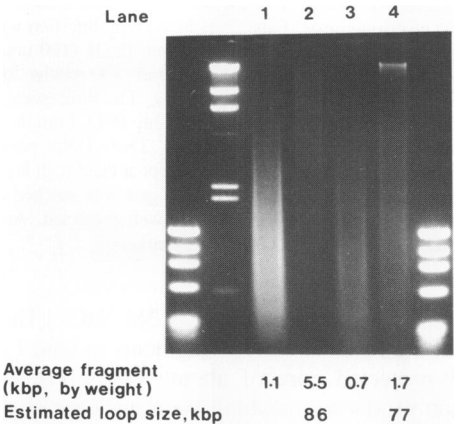


Fig. 5. Attached fragments are stably protected from digestion over a considerable length. [^{3}H] labelled cells were encapsulated (5×10^6/ml), lysed, washed, treated (32°C; 30min.) with *Hae*III (500 units/ml) plus *Eco*RI (2,500 units/ml) and detached chromatin removed by electrophoresis. Beads were recovered and retreated with a mixture of *Alu*I, *Rsa*I, *Sau*3A, *Mbo*I, *Msp*I, *Hpa*II, *Hin*fI and *Pst*I (each at 500 units/ml; 32°C; 30min.) and then detached chromatin electroeluted from beads in a dialysis bag. DNA was purified from both the beads (attached DNA) and the fluid in the bag (detached DNA). The sizes of DNA in samples withdrawn at different stages in the procedure were analyzed; after staining, the resulting gel was photographed and the average fragment and loop size determined. Lane 1: DNA fragments in total chromatin after treatment with *Hae*III plus *Eco*RI. Lane 2: DNA remaining in beads after the first electroelution. Lane 3: DNA in chromatin fragments electroeluted after the second digestion with the eight different enzymes. Lane 4: DNA remaining in beads after the second electroelution. 10^6, 2.5×10^6, 10^7 and 2.5×10^7 cell equivalents were loaded in lanes 1–4 respectively. The fluorescent material at the top of lane 4 contains little ^{3}H and is probably DNA from the 1–2% of dead cells always to be found in HeLa cultures. Their DNA proves resistant to digestion and generally goes undetected except when most DNA has been removed. Markers: outside lanes—phiX174/*Hae*III; second lane from left— λ/*Hind*III.

(Fig. 4, lanes 5 and 6). If histones were redistributing, sites would become exposed and the partial products would disappear. This experiment also confirms that nucleosomes are not 'phased' along the globin locus; particular sites are not accessible (or blocked) in all cells.

Attachments extend over many base-pairs

The residual fragments that remain are much larger than the fragments that can be removed. This can be demonstrated by treating beads with an excess of *Hae*III and *Eco*RI; this cuts DNA in total chromatin into 1.1kbp pieces (Fig. 5, lane 1). However, after electroelution, the remaining (attached) fragments are 5× larger (ie 5.5kbp; Fig. 5, lane 2). Further treatment of the beads containing this residual fraction with another eight different restriction enzymes then detaches a little more chromatin (number average size 0.42kbp; lane 3), but the resistant fraction is 0.7kbp larger (ie number average size 1.1kbp; lane 4). This resistant fraction is not larger because it lacks sites; it has the same size distribution as pure genomic DNA when both are cut under identical conditions (results not shown). Presumably these fragments are larger because sites within an attached region of 0.7kbp are protected from these nucleases. [Of course, as we are dealing with a minor fraction of DNA, it remains formally possible that this fraction cannot be cut or electroeluted for unknown reasons, despite its very small size.] This experiment also confirms how stable these attachments are; loop size remains essentially unchanged throughout the lengthy procedure (Fig. 5).

Some attachments are lost in 2M NaCl

The sensitivity of attached fragments to high concentrations of salt provide additional, although circumstantial, evidence for extended attachments; salt detaches some of the residual fragments. This can be shown by detaching chromatin as before, then treating the beads with different concentrations of NaCl; fragments detached by the salt were then removed electrophoretically and those remaining attached were sized on a gel (Fig. 6A). Increasing salt concentrations slightly and progressively remove DNA and it is the smaller fragments that are electroeluted preferentially (Fig. 6B,C). These effects, though small, are quite reproducible. [Note that in Fig. 8 the effect is undetected as the electrophoresis after 2M salt treatment was omitted.] They imply that the strength of attachment depends on length, presumably because increasing length allows cooperative—and stronger—interactions. These results are in keeping with earlier observations that showed that loop size increased by about a quarter when cells were lysed in 2M NaCl rather than the 'physiological' buffer (7) and that attachments resistant to 2M NaCl extend throughout actively-transcribed genes (2).

The size of the attached region

In principle, we can distinguish between one continuous attachment of about 1kbp and several adjacent but shorter attachments which together cover about 1kbp using endo- and exo-nucleases. Exonucleases should trim the ends of residual *Hae*III fragments to the outermost attachment point: endonucleases might cut within the mini-loops between a number of attachment points whilst leaving a continuous 1kbp attachment untouched. There are, however, a number of theoretical and technical problems with such an approach. For example, we require that the nucleases be active in the physiological buffer, with the exonucleases lacking endonuclease activity and the endonucleases unable to cut within protected regions. Unfortunately we could find none that fulfilled these criteria. For example, some exonucleases (ie Bal 31 and Mung bean nuclease) had sufficient endonuclease activity to convert supercoiled plasmid DNA to fragments of <500bp within 1h (results not shown) and some endonucleases (ie DNase and micrococcal

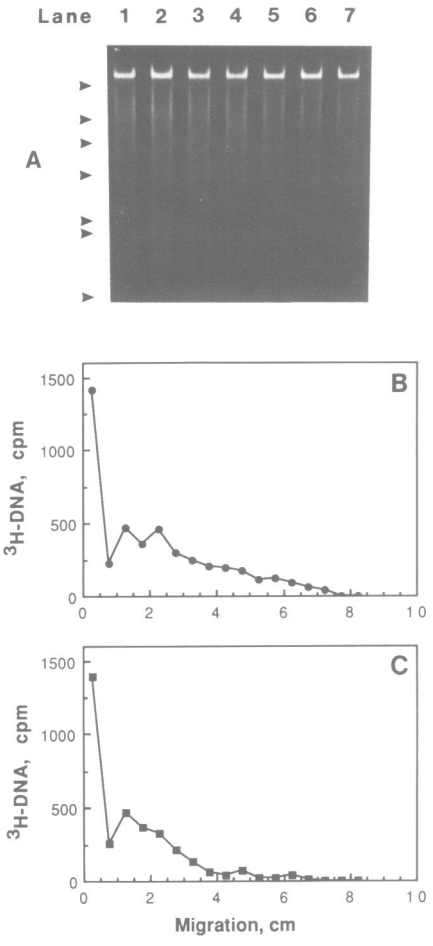


Fig. 6. Stability of attachments in high concentrations of NaCl. [^{3}H] labelled cells were encapsulated, lysed, treated with *Hae*III (750 units/ml; 32°C; 30min.) and detached chromatin removed by electrophoresis. Beads were recovered, incubated (30min. on ice) in modified buffers in which the monovalent ion concentrations were adjusted to 0, 0.15, 0.25, 0.5, 0.75, 1 or 2M with NaCl (lanes 1–7 respectively), washed in the physiological buffer and any released fragments removed electrophoretically. After re-recovering the beads, DNA was purified and applied to an analytical gel. (A) Photograph of the gel. 90, 100, 100, 92, 90, 88 and 85% ^{3}H remained after salt treatments and electrophoresis in lanes 1–7 respectively. Arrowheads: λ/*Hind*III markers. (B,C) ^{3}H distributions in lanes 2 and 7 respectively.

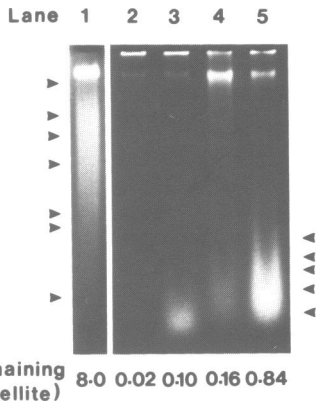


Fig. 7. The size of completely-protected fragments. [^{3}H] labelled cells were encapsulated (2.5×10^6 cells/ml), lysed, treated with *Hae*III (500 units/ml; 32°C; 30min.) and detached chromatin removed by electrophoresis. Beads were recovered. Some beads were washed in 2M NaCl (lanes 4 and 5) and samples re-incubated with different nucleases for 20h at 32°C. Detached fragments were then removed electrophoretically, residual fragments purified free of agarose and visualised after electrophoresis in agarose (1.5%, except for lane 1 with 0.8%). Lane 1: fragments remaining after *Hae*III cutting and the first electrophoresis. Lane 2: fragments remaining after the second nuclease treatment (DNase; 500 units/ml) and electrophoresis. Lane 3: as lane 2 but digestion with micrococcal nuclease (500 units/ml). Lane 4: as lane 2 with Bal31 (100 units/ml). Lane 5: as lane 2 with Mung bean nuclease (500 units/ml). The relative loadings of beads were 1:40:40:20:20 in lanes 1–5 respectively. The fluorescent material at the top of tracks 2–5 contains little ^{3}H. It is probably DNA from the 1–2% of dead cells always to be found in HeLa cultures. Their DNA proves resistant to degradation and generally goes undetected except at these high levels of digestion. Control experiments showed that the limit digest was reached after 1–3h, so that the residual fragments are clearly very well protected. Arrowheads: left, λ/*Hind*III markers; right, phiX174/*Hae*III markers.

nuclease) cut within complexes, eventually reducing DNA in chromatin to very small fragments (see later). In addition, both Mung bean and S1 nucleases are active only at low pH. Another problem is that exonucleases only trim back to the first nucleosome unless histones are removed; although this can be easily achieved by hypertonic treatment, this inevitably changes loop size slightly (7) and destroys some attachments (Fig. 6). Furthermore, kinetic experiments of the kind often used with pure DNA-protein complexes prove difficult to interpret when many different complexes are present.

Nevertheless, these enzymes do allow us to demonstrate that the residual attached sequences are very well protected from nucleolytic attack (Fig. 7). Digestion with large amounts of DNase for 20h, followed by electrophoresis, effectively removes all DNA (Fig. 7, cf lanes 1 and 2; note that 40× more beads were loaded in lane 2). Micrococcal nuclease (lane 3) and the exonucleases Bal31 and Mung bean nuclease (lanes 4 and 5) do, however, leave some small pieces attached. [Note that histones were stripped from the residual fragments in the samples analyzed in lanes 4 and 5 by pre-treatment with 2M NaCl.] This experiment shows that the residual *Hae*III fragments in lane 1 are trimmed to a well-protected core of about 500bp. For example, the distribution produced by Mung bean nuclease (Fig. 5, lane 5) has a weight average size of 0.86kbp (number average, 0.5kbp). This must be seen as a lower limit of the size of the attached region, as the hypertonic treatment destroys some attachments (Fig. 6) and the exonucleases cut to some extent within the attached regions.

The size of fragments resistant to exonuclease III

We also investigated the size of the attached region using exonuclease III. Preliminary experiments showed that this exonuclease was active in our buffer and freer than others from endonuclease activity. In principle, the protected region can be sized as follows. Beads are first treated with *Hae*III and unattached chromatin removed electrophoretically. Then histones are stripped from the residual fragments and exonuclease allowed to degrade from the 3'ends of the attached fragments to the first point of attachment. Finally the single-strands that extends from the attachment point are trimmed with a single-strand specific nuclease. We chose to use Mung bean nuclease, rather than S1 nuclease, as it is active at a pH which does not destroy attachments (ie pH 6.5, see above) and which is closer to the physiological than that required by S1 nuclease. Double-stranded products from exonuclease III cannot be sized accurately on non-denaturing gels because they are partly single-stranded and so have mobilities and ethidium-binding properties unlike fully double-stranded molecules. Therefore the products were also analyzed as single-stranded molecules after denaturation. This approach has the

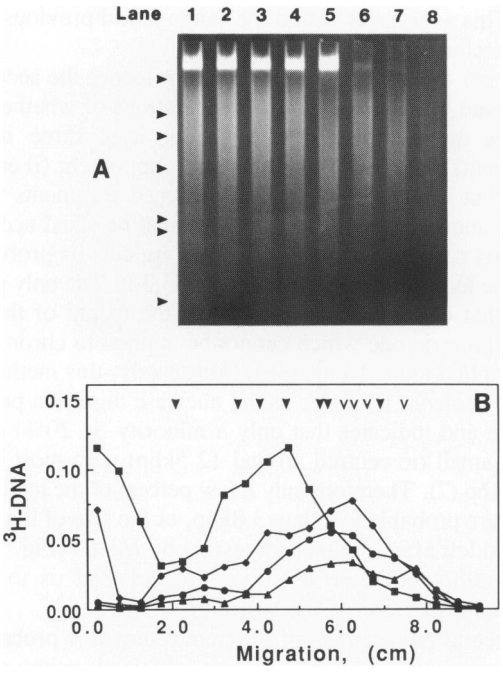


Fig. 8. The size of the attached region determined using exonuclease III. [^{3}H] labelled cells were encapsulated (5×10^6 cells/ml), lysed, treated with *Hae*III (500 units/ml; 32°C; 30min.) and detached chromatin removed by electrophoresis. Beads, containing 6.1% chromatin, were recovered; half were washed in 2M NaCl (lanes 5−8) to remove histones and returned to isotonic conditions. Samples were incubated (32°C; 30mins) with different concentrations of exonuclease III, washed to remove solubilised material and any fragments remaining in beads released by treatment with SDS and proteinase K. The photograph in A illustrates the sizes of double-stranded fragments after electrophoresis. Other samples were also treated with Mung bean nuclease, denatured and the size of residual single-stranded fragments determined using agarose gels (B). (A). The effect of exonuclease III after removing histones with 2M NaCl. Lanes 1−4: control samples not washed in 2M NaCl and treated with 0, 250, 1000 or 2500 units/ml exonuclease III (lanes 1−4 respectively). Lanes 5−8: samples washed in 2M NaCl and treated with 0, 250, 1000 or 2500 units/ml exonuclease III (lanes 5−8 respectively). Arrowheads: λ/*Hind*III markers. (B). The size of single-stranded fragments resisting exonuclease III and Mung bean nuclease treatment. All samples were denatured with 0.2M NaOH before electrophoresis in 1.2% agarose; the lanes were excised and sliced, and the [^{3}H] in each slice expressed as a fraction of the total in the lane containing 6.1% chromatin (squares). Squares: fragments from beads containing 6.1% chromatin that resisted electroelution after *Hae*III treatment; double-stranded DNA from the same sample was analyzed in A, lane 1. Diamonds: fragments resisting electroelution, treatment with 2M NaCl to remove histones and exonuclease III; double-stranded DNA from the same sample was analyzed in A, lane 8. Circles and triangles: fragments resisting electroelution, treatment with 2M NaCl, exonuclease III and 100 (circles) or 500 (triangles) units/ml Mung bean nuclease. Weight averages and recoveries were 5.3kbp and 100%, 3.3kbp and 66% (diamonds), 2.1kbp and 38% (circles) and 1.4kbp and 30% (triangles). Closed arrowheads−λ/*Hind*III markers; open arrowheads−phiX174/*Hae*III markers.

general drawbacks that unphysiological conditions are used for histone removal (ie 2M NaCl) and Mung bean nuclease treatment (ie pH 6.5 and the presence of Zn^{2+}).

Fig. 8A illustrates the analysis of undenatured DNA. Beads were first treated with *Hae*III and unattached chromatin removed electrophoretically. As expected, incubation with exonuclease III then has little effect on the size of the attached fragments (lanes 1−4); the enzyme can only degrade from the cut 3′ ends back to the nearest nucleosome. However, if nucleosomes are removed, the picture is different (lanes 5−8). High concentrations of exonuclease III removed most of the *Hae*III satellite; degradation from each 3′ end of these long double-stranded fragments converts them largely to single-stranded fragments that stain inefficiently. High concentrations also degrade slightly the attached fragments in the middle of the gel, decreasing their apparent length (number average) from 3.1kbp (lane 5) to 2.3kbp (lane 8). As these are much shorter than the satellite that is completely degraded, exonuclease action on them must be blocked. [Note that long incubations with the highest concentration of nuclease do eventually reduce the size of all DNA fragments, presumably due to endonucleolytic cleavage. Note also that it remains formally possible, though unlikely, that exonuclease III degrades the satellite to moderately-sized fragments that, for unknown reasons, cannot be degraded further.]

Fig. 8B illustrates sizes after denaturation. The residual *Hae*III fragments analyzed in Fig. 8A, lane 5, as double-stranded molecules are of two types, derived from the satellite (in the first gel slice) and most of the attached DNA (Fig. 8B, squares). The highest concentration of exonuclease III used in Fig. 8A, lane 8, only slightly degrades these (Fig. 8B, diamonds), confirming that enzyme progression must be blocked in both fractions. Trimming their single-stranded ends with Mung bean nuclease then completely eliminates the satellite fraction but leaves a considerable amount of material in the middle of the gel (Fig. 8B, circles and triangles). Clearly, there is sufficient nuclease to completely degrade the long satellite—which again provides an internal control—and the higher concentration probably begins to degrade the resistant fraction in the middle of the gel by endonucleolytic cleavage (Fig. 8B, triangles).

These results are consistent with the following model. Treatment with *Hae*III followed by electroelution leaves fragments of 3.1kbp at the bases of the loops; 0.7kbp within this is protected from nucleolytic attack, leaving 1.2kbp on each side organised into nucleosomes. When histones are removed, exonuclease III degrades one third of the DNA (ie 1200 nucleotides from each flanking region). Trimming with Mung bean nuclease leaves a resistant core—the attachment region—of about 0.75kbp, constituting 25% of the mass of the original 3.1kbp fragment. The observed values are in remarkable agreement with this model (Fig. 8B, legend), but are subject to the reservation that unphysiological conditions were used. Whatever the precise explanation, this experiments confirms that an extended region of about 1kbp at the base of the loops is protected from exonucleolytic attack.

Sensitivity of attachments to ionic detergents

Essentially all the residual fragments can be detached by SDS (Fig. 9, cf lanes 1 and 6; note that the satellite remains too large to be electroeluted from beads by the conventional electrophoretic field), showing that they are not irreversibly fixed in beads. Sarkosyl detaches some fragments but not others (lanes 2 and 3), suggesting there may be different types of attachment.

DISCUSSION

Potential artifacts

As artifactual aggregates are often formed non-specifically, specificity of attachment of a sequence to a structure found *in vitro* provides one good criterion that it has a counterpart *in vivo* (9). However, such specific interactions have generally been demonstrated in structures isolated using unphysiological conditions and, in every case, the criticism that they are artifactual aggregates remains difficult to rebut. To cite one example from

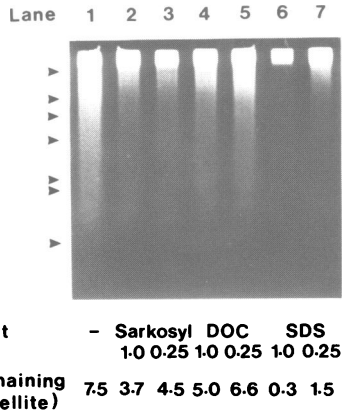


Fig. 9. Sensitivity of attachments to detergents. [^{3}H] labelled cells were encapsulated, lysed, treated with *Hae*III (500 units/ml), detached chromatin removed by electrophoresis and beads recovered. Identical samples were incubated (15min; 0°C) with N-laurylsarcosine (sarkosyl), deoxycholate (DOC) or sodium dodecyl sulphate (SDS) at the indicated concentrations, washed and detached fragments removed electrophoretically. Beads were recovered and remaining DNA purified and visualised after electrophoresis. A control with no detergent extraction is shown in lane 1. Arrowheads: λ/*Hind*III markers.

our own work, replicating and transcribing regions are specifically attached in nucleoids (isolated in 2M NaCl), but such active chromatin is rich in single-stranded nucleic acids which might be expected to aggregate into a larger structure (for a review, see ref. 2). Then, it is not surprising that nascent RNA and DNA is found associated with the structure *in vitro* or, as only some sequences are transcribed, that attachments are specific. The same criticism applies to matrices (3), which are additionally exposed to the hypotonic conditions known to specifically aggregate ribonucleoprotein particles into filaments (30). The most detailed mapping has been carried out on scaffolds (8), in which topoisomerase II consensus sequences are tightly attached (31,32; see also ref. 33). However, as five out of every six attachments in scaffolds are probably generated during the incubation necessary to 'stabilise' the structures (7, 21–24), it remains to be seen whether such intellectually pleasing attachments have counterparts *in vivo* (but see ref. 34).

As the history of sub-nuclear structures seems to be a history of artifacts, the results here must obviously be treated cautiously. However, we have used conditions throughout that resemble as far as is conveniently possible those *in vivo*. The major difference between our buffer and the *milieu interieur* is that the buffer contains Cl^-, and not protein, as the major counterion, but we know of no artifacts that this might cause. The Triton used for lysis is probably the constituent most likely to generate artifacts by destroying pre-existing structures. Although similar detergents give similar results, they might all be having the same destructive effects. Any biochemical approach is inevitably a destructive one, but this is the gentlest that we can devise, with chromatin being removed by a combined nuclease treatment and electrophoresis. Perhaps the best evidence that a structure survives the treatment free of artifact is circumstantial—function is preserved. It is difficult to imagine that major structural rearrangements occur whilst essentially all the replicational and transcriptional activity of the living cell is retained during lysis, nuclease treatment and electroelution (15).

Loop size

Using various enzymes and different levels of detachment, we reproducibly find a loop size of 80–90kbp. This is, of course, an average; a wide range of differently-sized loops probably exists (7,35). This average is within the range found previously using various techniques (3, 18, 35–40).

A number of technical factors could influence the accuracy of our estimate, quite apart from considerations of whether or not the loops are artifacts. There are at least three technical requirements that must be met using this approach; (i) each loop must be cut at least once, (ii) all detached fragments must be removed and (iii) residual fragments must be sized accurately.

The first requirement—that all loops are cut—is probably not met; some loops may be very small (7,35,36). The only practical method that can be used to determine the extent of this range is a fluorometric one which cannot be applied to chromatin but can be applied to nucleoids (36). Fortunately, this method gives the same nucleoid loop size as the nuclease digestion procedure used here and indicates that only a minority (ie 20%) of loops are very small (ie centred around 12.5kbp) with most between 50–250kbp (7). Therefore only a few percent of the total number of loops are probably less than 3.8kbp, or the size of the residual chromatin left after complete digestion by *Hae*III (Fig. 1B, lane 4)). Nevertheless, these few loops would lead us to slightly overestimate the size of the majority.

The second requirement of efficient removal is probably met as the same average loop size is obtained using different detergents, buffers and pHs, as well as different enzymes, levels of digestion and electrophoretic conditions. Inefficient removal would lead to an underestimate of loop size. Similarly, the third requirement is met using *Hae*III and conventional fields for electrophoresis.

Attachments are very stable

Perhaps our most striking finding is how stable chromatin and the attachments prove to be in our buffer. We have no evidence for any nucleosomal 'sliding' during long incubations (Fig. 4) and the attachments survive repeated nuclease digestions and electrophoresis (Fig. 5). Some survive treatment with 2M NaCl or sarkosyl (Figs. 6 and 9) suggesting there may be different types of attachment. We hope that these differences in strength of attachment will help us identify the proteins and sequences at the various attachment sites.

The size of the attached region

Complete digestion with *Hae*III cuts encapsulated chromatin into 1.7kbp pieces, consistent with cutting between nucleosomes but not within them. After electroelution, the residual fraction is larger (ie 3.7kbp; Fig. 1B, lane 4), suggesting that extra sites within an attached region of 2kbp are protected from the nuclease. More extensive digestion with a number of different restriction endonucleases eliminates any asymmetries introduced by *Hae*III cutting and leaves a smaller residual fraction of 0.7kbp (Fig. 5).

Protection over such extended regions can be explained by: (i) attachment continuously throughout 0.7kbp to the skeleton; (ii) several shorter attachments, which together cover 0.7kbp, spaced over a longer distance; (iii) a point attachment embedded in 0.7kbp of chromatin which has such an altered structure that all restriction sites within it are inaccessible; (iv) a combination of these. Model (ii) is consistent with the mixture of 8 different restriction enzymes giving a smaller protected region than *Hae*III alone (Fig. 5). In principle, these models can be distinguished using endo- and exo-nucleases, but contaminating activities made analysis difficult. [The unphysiological conditions required for their use (ie 2M NaCl and pH 6.5) also compromise results obtained with them, but control experiments showed that these

only distorted attachments slightly (Fig. 6).] Nevertheless, a region of 500bp long within most attached fragments was resistant to Bal31 or Mung been nuclease (Fig. 7); the resistant region may be larger than this as these enzymes had some endonuclease activity. A sequential treatment with exonuclease III and Mung bean nuclease confirmed that the protected region was about 0.75kbp long (Fig. 8).

This length of about 1000bp is much longer than that of enhancers or topoisomerase consensus sequences that have been canvassed as attachment sites. Whilst models with essentially point attachments (ie involving one, or a few, protein-binding sites of 10–20bp) have received considerable attention, those involving extended attachments have received little (however, see 41–43) but seem to have much to commend them. If many relatively low-affinity sites of 10–20bp together cover up to 1kbp and stabilise attachments, the strength of attachment would depend on length. This is what is found: small fragments are detached preferentially by 2M NaCl (Fig. 6C). A large number of low-affinity interactions would also allow some to be disrupted as polymerases transcribed or replicated within attached regions, without overall attachments being lost. This is consistent with a generalised attachment of active genes that we have found in this material (15,20).

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