THE ATTACHMENTS OF CHROMATIN LOOPS TO THE NUCLEOSKELETON

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ABSTRACT

It is widely assumed by cell biologists that chromatin is looped by attachment to some nuclear skeleton. 'Structural' attachments might be mediated through specific sequences; these would be attached in most cells in an organism, underlying the basic structure of the mitotic chromosome and persisting throughout interphase. 'Functional' attachments might also exist, perhaps if active polymerases are attached to the skeleton and replication and transcription occur as DNA is reeled through them. Cells of different tissues - and even cells of the same tissue - would have different attachments of this type. Problems associated with demonstrating these two kinds of attachment are discussed. We find little good evidence for 'structural' attachments and explore the idea that 'functional' attachments are the only kind that exist: 'functional' attachments involving active transcription units might be stable enough to organize chromatin during both interphase and mitosis, but 'dynamic' enough to allow duplication of attached sequences without disrupting loops.

INTRODUCTION

A fundamental belief in cell biology is that chromosomal DNA is looped into domains by attachment of the chromatin fibre to a nucleoskeleton. Whilst cell biologists might argue about which DNA sequences are responsible for attachment and what the precise molecular constitution of the nucleoskeleton might be, they are agreed that some structure must ramify throughout the interphase nucleus, organizing the chromatin fibre to ensure it can be correctly duplicated and segregated to daughter cells. Different minorities amongst cell biologists also attribute functional, as well as structural, roles to this skeleton. Some see it as the active site of replication or of the repair of DNA damage, others of transcription, RNA processing or transport. Indeed, a pivotal role for a skeleton has been invoked by different cell biologists for almost every nuclear process (Nelson et al., 1986).

Another view - crudely described as that of the molecular biologist - lies in stark contrast. Their descriptions of replication and transcription involve diffusible polymerases acting upon a chromatin fibre devoid of higher-order structure above the level of the solenoid. If pressed, they might concede that a skeleton plays a structural or packaging role, but most deny any functional role during replication and transcription. They dismiss the evidence for such a role - the association of some skeleton with nascent nucleic acids or the relevant polymerases - as an artefact generated by cell biologists after lysing the cell: polymerases and nascent nucleic acids are sticky and could be expected to co-aggregate - perhaps quite specifically -

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with different elements in the dense chromatin (for example, see Martelli et al., 1990). Such views are confirmed by the obvious disagreements amongst the cell biologist who, by varying their isolation procedures, isolate very different structures (e.g. external lamins or internal 'matrix'). [See Belgrader et al. (1991) for a recent review of matrix structure and Cook (1988) for a review of the controversy].

If any consensus is to be drawn from these different views, it is probably that some skeleton probably does package the chromatin fibre. Such 'structural' attachments would persist throughout the life of any one cell, with similar attachments being found in most cells in an organism. They would underlie the basic structure of the mitotic chromosome and persist throughout interphase. They can be differentiated from the controversial 'functional' attachments, which - if they exist - must be dynamic in the sense that the attached sequence continually changes if DNA is reeled through an attached polymerase during replication and transcription.

We review here evidence for different attachments. Our viewpoint is that of a laboratory that initially demonstrated supercoiling in the looped domains of nuclear DNA and subsequently showed that the size of these loops remained constant as cells progressed around the cell cycle, implying the existence of 'structural' attachments. We then showed that sequences that were being replicated, transcribed and repaired were also attached, suggestive of different 'functional' attachments (reviewed by Jackson et al., 1984). But these experiments all involved extracting cells in 2 M NaCl and so could be dismissed on the grounds that the attachments were artifacts created during isolation. To answer such criticisms we have confirmed using physiological conditions that replicating and transcribing DNA are attached (reviewed by Cook, 1989, 1991). Our perspective, then, is coloured by this history.

As implied above, there is little agreement as to the nature of any nucleoskeleton. Controversy centres on whether candidate structures (e.g. matrices, scaffolds, cages) are isolation artifacts, with no counterparts *in vivo* (Cook, 1988). Therefore, we concentrate on attachments seen *in vivo* or in the few studies involving physiological conditions. Fortunately, meiotic lamp brush chromosomes of living newt cells do provide undisputable proof of chromatin loops attached to a skeletal core (Callan, 1977). An intermediate-filament-like skeleton is also seen using a 'physiological' buffer (Jackson and Cook, 1988; see also He et al., 1990; Wang and Traub, 1991). Structures like matrices, scaffolds and cages all have very different loop sizes - and hence attachments - highlighting how real the problem of artifacts is (Jackson et al., 1990).

DEFINING ATTACHED SEQUENCES

Three methods have been used widely to define attached sequences. The first involves progressively detaching DNA from the skeleton with a nuclease; sequences close to attachment sites should resist detachment and so be enriched in a pelleted fraction: those lying further away should be depleted. If attachments are completely random, there should be no specificity in the aggregate and any given sequence will neither be enriched nor depleted. In fact, α globin sequences in HeLa nucleoids can be enriched 8x whereas β and τ genes are depleted; α globin must lie closer to the attachment site than β or τ. This 'detachment mapping' approach was extended to the

integrated genes of polyoma and Rous sarcoma virus; active genes, but not their inactive counterparts, were closely associated with the nucleoid 'cage' (reviewed by Jackson et al., 1984). Enhancer regions were particularly closely associated. This method has now been applied to many different preparations (e.g. matrices and scaffolds), but with the variable results expected if each preparation had its own set of artificially created attachments. Indeed the criticism that the attachments seen in such preparations is difficult to rebut. To cite one plausible explanation of our own results, replicating and transcribing regions might become specifically attached in nucleoids (isolated in 2 M NaCl) because active chromatin is rich in single-stranded nucleic acids which might be expected to aggregate into a larger structure. Then, inevitably nascent RNA and DNA would associate with the structure *in vitro* and, as only some sequences are transcribed, the attachments seen would be specific. The same criticism applies to matrices, which are additionally exposed to the hypotonic conditions that create another set of artefactual attachments (Jackson et al., 1990).

One particular set of attachments defined in this way are the 'scaffold attachment regions' or SARs (e.g. Amati and Gasser, 1988; Bode and Maass, 1988; Klehr et al., 1991; Brun et al., 1990). They are remarkable in that essentially all of the SAR partitions with the pelletable material in the assay, rather than a significant fraction (as is usually the case with matrices or nucleoids). SARs often contain the consensus sequence for topoisomerase II, a structural component of scaffolds in which they are found (Berrios et al., 1985; Earnshaw et al., 1985). However, there are powerful reasons for believing that these intellectually-pleasing attachments are generated *in vitro* during isolation. First, no scaffold-like structure can be obtained after extraction with the detergent (i.e. LIS) used to isolate the scaffolds unless it is first created *in vitro* by a thermal 'stabilization' step (Mirkovitch et al., 1984; Cardenas et al., 1990). Such a step is known to induce a heat-shock-like aggregation of protein (Evan and Hancock, 1987; McConnell et al., 1987; Berrios and Fisher, 1988; Cardenas et al., 1990; Kaufmann and Shaper, 1991). It also generates five new (artefactual) attachments in HeLa nuclei for every one that pre-existed (Jackson et al., 1990). Even more suggestive of an artefact is the observation that glutaraldehyde, far from fixing the structure, actually prevents scaffolds from forming (Mirkovitch et al., 1984). Moreover, SARs play no detectable role in packaging chromatin in nuclei (Eggert and Jack, 1991). Therefore, what relationship these fashionable isolates bear to any structure *in vivo* is open to argument; they may simply reflect the fact that a sticky topoisomerase inevitably binds its target sequence during incubation *in vitro*. There is also no particular reason to believe results obtained with scaffolds any more than the very different results obtained with other structures like matrices and nucleoids.

Attachment sequences can also be selected by incubating nuclei with DNA fragments to see which bind specifically (Cockerill and Garrard, 1986; Cockerill et al., 1987). Little specificity is seen unless nuclei are first extracted with 2 M NaCl or heat-shocked and treated with lithium diiodosalicylate; then fragments containing enhancers or topoisomerase II sites bind specifically. Such sequences - initially called matrix attachment regions or MARs - have some similarity to SARs. However, it is difficult to know whether such complexes are analogous to those *in vivo* or artifacts due to aggregated topoisomerase trapping its consensus sequence. It seems unlikely

that these sequences could be 'structural' attachments, as the canonical MAR in the immunoglobulin locus lies within a transcription unit. Moreover, this MAR has no significant function when assayed in transient expression assays or in transgenic mice (Blasquez et al., 1989; Xu et al., 1989).

Attachment sequences might also be defined functionally. Insertion of exogenous genes into chromosomal DNA generally leads to variable expression of the inserted gene, depending on the particular chromosomal insertion site. Such position effects can be overcome if locus control regions (LCRs), like those at the ends of the globin, lysozyme and interferon-β loci, are co-inserted with the gene. The LCR prevents the chromosomal position effects, allowing tissue-specific expression of the inserted loci (Grosveld et al., 1987; Stief et al., 1989). Most interestingly in the present context, the LCR is highly transcribed (Collis et al., 1990) and its sequence contains binding sites for classical transcription factors (Philipsen et al., 1990; Talbot et al., 1990). The particular region of the lysozyme locus that turned out to be the LCR was initially chosen for analysis because it was attached to the matrix and recently a protein binding to it has been identified (Von Kries et al., 1991). Similarly, the region in the interferon-β locus was first identified as a SAR (Klehr et al., 1991). A genetic assay for sequences that prevent position effects is also available in *Drosophila* (Kellum and Schedl, 1991; see also Gyurkovics et al., 1990). It is attractive to suppose that these sequences that buffer the inserted gene from position effects are the points of attachment at the boundaries of a chromatin domain and that these functional assays will turn up more attachment sequences. But although the functional effects of these regions are remarkable, it remains to be demonstrated that any of them are associated with a skeleton *in vivo*.

ATTACHMENTS SEEN USING PHYSIOLOGICAL CONDITIONS

Unphysiological salt concentrations are almost universally used to isolate nuclei because chromatin tends to aggregate under isotonic conditions. Aggregation can also be suppressed by high concentrations of magnesium ions, but these activate nucleases and irreversibly fix the chromatin (see below). However, problems of aggregation can be sidestepped by encapsulating HeLa cells in agarose microbeads (r = ~25 μm) before lysing membranes with Triton X-100 in a 'physiological' buffer (Jackson and Cook, 1985; Jackson et al., 1988). [The buffer (pH 7.4) we currently use 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.] This buffer preserves gross structure and maintains DNA integrity (assayed by the presence of supercoiling after removing histones): function is also preserved since such encapsulated nuclei synthesize RNA and DNA authentically at the rate found *in vivo*. This material is not subject to a heat-shock-like aggregation induced by incubation at 37°C. In these isolates attachments are also extremely stable; the measured loop size remains constant during isolation and repeated pelleting and resuspension. There is also no evidence of nucleosomal 'sliding': restriction yields specific partial digestion products that persist during long incubations, showing that 'sliding' cannot be uncovering new restriction sites (Jackson et al., 1988; 1990b). To our knowledge, no other preparation preserves both structure and function to this extent. Most importantly, this encapsulated

material is freely accessible to molecular probes (e.g. enzymes, antibodies) yet can be pelleted and resuspended repeatedly without aggregation.

As the history of sub-nuclear structures seems to be a history of artifacts, any claim that a preparation is relatively free of artefactual attachments must obviously be treated cautiously. But here function is preserved: it is difficult to imagine that major structural rearrangements could occur whilst essentially all the replicational and transcriptional activity of the living cell is retained, especially when attachments involve active polymerases.

'Detachment mapping' has been used to define the attached sequences in this material. Most chromatin can be removed by treatment with an endonuclease followed by electrophoresis to leave residual clumps of chromatin attached to an intermediate-filament-like skeleton (Jackson and Cook, 1988). Despite removal of most chromatin, essentially no nascent RNA or DNA - whether synthesized *in vitro* or *in vivo* - is lost. This implies that at least some of the attachments in this material are 'functional' and involve replicating and transcribing sequences. Essentially all DNA and RNA polymerizing activity is also retained, presumably because the relevant polymerases are attached to some larger structure (Cook, 1989; 1991). The recovery of essentially all activity and not a minor fraction, makes explanations based on an artefactual aggregation of polymerases or nascent nucleic acids difficult to sustain; such an aggregation would be expected to reduce polymerizing activity.

An average loop size of 86 kb (in HeLa cells) can also be determined from the percentage of chromatin remaining in the beads after electro-elution and the size of the attached fragments (Jackson et al., 1990). This loop size can be changed from 15-125 kb by only slight departures from the physiological. The 'mild' hypotonic conditions generally used to isolate nuclei halve loop size; this means that for every attachment existing *in vivo*, one new attachment is created *in vitro* as nuclei are prepared. High concentrations of magnesium ions also irreversibly fix the chromatin into small loops (i.e. create new attachments). [Note that we generally chelate magnesium ions with an equal concentration of triphosphate.] Structures like matrices and scaffolds - which are derived from such nuclei - have accumulated additional rearrangements. Even though it has been argued that binding of specific sequences to these structures implies that attachments are not created artefactually it seems that most of them arise specifically after lysis. For example, five out of every six loops in 'LIS'-extracted scaffolds are seen only after the thermal 'stabilization' step.

The loop size measured under physiological conditions remains unchanged throughout the cell cycle. Therefore the fundamental attachments probably persist during the gross structural changes occurring during mitosis. Loop size measured in this way is, of course, an average. [For a discussion of loop sizes, see Jackson et al. (1990).]

Given that chromatin is poised in a metastable state and very prone to rearrangement, we were surprised how stable attachments proved to be in our buffer (Jackson et al., 1990b). They survive repeated nuclease digestions and electrophoresis. Some even survive treatment with 2 M NaCl or sarkosyl - perhaps the sub-fraction involving transcription complexes that cannot be disrupted by this agent. Perhaps even more surprising was the size of the attached region. Complete digestion

with HaeIII cuts encapsulated chromatin into 1.7 kb pieces, consistent with cutting between nucleosomes but not within them. After electro-elution, the residual fraction is larger (i.e. 3.7 kb), suggesting that extra sites within an attached region of 2 kb are protected from the nuclease. More extensive digestion with a number of different restriction endonucleases and/or exonucleases eliminates any asymmetries introduced by HaeIII cutting and leaves a smaller residual fraction of ~0.7 kb. Such a length for an attached region is much longer than point attachment sites of topoisomerases that have been so widely canvassed. However, a large number of relatively low-affinity sites which together cover up to 1 kb and stabilise attachments would allow some to be disrupted as polymerases transcribed or replicated within attached regions, without overall attachments being lost.

ARE 'STRUCTURAL' AND 'FUNCTIONAL' ATTACHMENTS EQUIVALENT?

All our experiments on encapsulated cells - as well as our earlier results on 'nucleoids' - are most simply explained if the relevant polymerases are attached to a skeleton, with the template moving through the polymerizing complex as nascent nucleic acids are extruded. These, then, are all attachments involving 'functional' sequences (i.e. transcribed regions, enhancers, replicating sequences). They will be dynamic in the sense that they depend on which particular part of the genome is being replicated or transcribed at any particular moment.

What, then, of the 'structural' attachments that most believe exist? To our great chagrin, we have been unable to uncover any sequence that completely resists electro-elution in all encapsulated cells in the population. This raises the questions: Do 'structural' attachments indeed exist *in vivo*? Can 'functional' attachments serve as 'structural' attachments? Perhaps surprisingly, separate pieces of evidence suggest that the elusive 'structural' attachments are, in truth, 'functional' attachments. Each piece is, by itself, hardly convincing but in aggregate, they allow a tentative case to be built.

First, we have failed to identify any 'structural' attachment sequences, despite much searching. The many more workers on the matrix have also been unable to identify any. Second, early work on nucleoids yielded some evidence that 'structural' attachments did not exist. Of course, this work is subject to the criticism that unphysiological salt concentrations were used, but we have subsequently shown that the attachments seen in HeLa nucleoids are similar, both in quantity and quality, to those found under physiological conditions. We might expect that transcriptionally and replicationally inert cells (i.e. hen erythrocytes and human sperm) might lack 'functional' attachments and contain only 'structural' attachments. However, of the wide range of different cells from higher eukaryotes (e.g. fibroblasts, lymphocytes, hepatocytes, teratocarcinoma cells from man, mouse, bird and insect) studied, only the functionally inert hen erythrocyte and human sperm failed to yield superhelical loops attached to a nucleoid 'cage'; instead lysis releases unstructured and relaxed DNA. Only they appeared to contain no attachments. Moreover, as the chicken erythroblast matures into the inert erythrocyte, replicational and transcriptional activity is lost concurrently with this loss of attachments (Cook and Brazell, 1976). And when the inert erythrocyte nucleus is reactivated on fusion with a fibroblast, a

matrix reforms (Woodcock and Woodcock, 1986). So in those cells where we might expect to see 'structural' attachments free of obscuring 'functional' attachments, none are found.

The structure of the bacterial nucleoid provides a third pointer to a sole role for 'functional' attachments. [Again these nucleoids are isolated using 2 M NaCl, so 'structural' attachments might be lost.] Their attachments involve functional RNA polymerases which contract the circular chromosome into loops (Krawiec and Riley, 1990).

Finally, the canonical LCR - the sequence that on functional grounds might be expected to be an attachment point in the β-globin locus - turns out to be highly transcribed (Collis et al., 1990) and contains binding sites for classical transcription factors (Philipsen et al., 1990; Talbot et al., 1990). Perhaps transcription and so attachment of the LCR precedes tissue-specific globin expression.

TRANSCRIPTION UNITS AS ATTACHMENT POINTS

Nature faces a dilemma when designing a 'structural' attachment point, defined as above. At some stage it must be duplicated, but it is difficult to see how this can be done without destroying attachments; we expect the attachments to be both stable enough to maintain the loops during all phases of the cell cycle but not so stable that they cannot be duplicated during S-phase, when presumably they must be disrupted. RNA polymerases may be uniquely designed to resolve this dilemma by providing both a stable and a moving attachment; attachment at the 3' end of the transcription unit could persist whilst the 5' end is replicated. Then we might imagine the template sliding through attached transcription and replication complexes. Once the promoter has been replicated, it will remain close to an RNA polymerase and could re-initiate by re-attaching. In this way attachments would be maintained at some point in the transcribed region, even during replication. Then, of course, the RNA polymerase-template attachments must persist through mitosis (when transcription ceases) as loops also persist.

As no role has yet been ascribed to the majority of nuclear transcripts - they do not seem to code for protein - it becomes attractive to suppose that they form these attachment points. Then it would be the transcribed repeats (perhaps Alu repeats) that punctuate the genome of higher eukaryotes that might be the elusive attachment points.

CONCLUSIONS

It has been assumed that two kinds of attachment of chromatin to the nucleoskeleton might exist - 'structural' and 'functional' attachments. 'Structural' attachments are seen as being stable, involving the same sequence in most cells in the organism. Such attachments would probably underlie the basic structure of the mitotic chromosome. Additional 'functional' attachments would involve active polymerases; as these are attached to the skeleton, which particular sequence became associated at any one time with the skeleton would depend on how far replication or transcription had progressed. As we can find little good evidence for 'structural' attachments, we have explored the idea that 'functional' attachments might be the only kind that exist.

Although there is no decisive evidence for this view, we think it warrants consideration. Such 'functional' attachments might allow resolution of the dilemma of how attachments might be stable enough to persist throughout the life of the cell, whilst being sufficiently transient to allow attached sequences to be replicated.

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