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DNA adjacent to attachment points of deoxyribonucleoprotein fibril to chromosomal axial structure is enriched in reiterated base sequences

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ABSTRACT

Mitotic chromosomes of L cells (metaphase plates) were dehistonized by centrifugation through a layer of 2 M NaCl and then treated with restriction endonuclease Bam HI. Alternatively, they were pretreated with EcoRI endonuclease, dehistonized, and additionally digested with EcoRI or HindIII. The DNA remaining attached to the axial structure of the chromosomes was isolated and investigated in renaturation experiments. It was found to be enriched in reiterated base sequences belonging to the satellite and to abundant intermediate repeats. The CsCl density gradient ultracentrifugation of this DNA separated the satellite from the fraction containing intermediate repeats.

INTRODUCTION

Studies on the electron microscopy of isolated cell nuclei treated by DNAase and/or salt solutions of different concentrations suggested the existence of axial structures in chromatin to which DNA·protein fibrils were attached /1/. The most clear-cut evidence for the presence of axial structures was obtained with histone-depleted mitotic chromosomes /2,3/. In these experiments /2,3/ also the long chromomere-size (50-100 kb) DNA loops whose existence was suggested earlier on the basis of biochemical data /4-6/ were visualized. The important question is whether the DNA·protein fibril is attached to the axial structure randomly or through specific DNA sequences. To answer this question, we prepared the histone-depleted mitotic chromosomes according to a modified Laemmli technique /2/, treated them with a restriction endonucleases, and analyzed the sequences remaining bound to the chromosomal axis in renaturation experiments. They were found to be enriched in reite-

rated base sequences including the satellite and intermediate repeats.

MATERIALS AND METHODS

Cell synchronization. L-cells 929 were grown in a monolayer upto the 20% covering of the surface. Then the complete Eagle medium was replaced for the methionine-free Eagle medium containing 10% calf serum. [^{3}H] thymidine (0.1 μCi/ml) and [^{35}S] methionine (0.1 μCi/ml) were added one-two generations prior to collection of the cells. 12 hr before this, the cells were transferred into the complete Eagle medium containing 10% calf serum and colcemide (0.05-0.1 μg/ml) (Gibco).

Mitotic cells were separated from interphase cells on the basis of their different adhesive ability. The percentage of mitotic cells among the cells removed from the monolayer was determined with the aid of fluorescence microscopy. Usually it was as high as 90-95% or even more.

Isolation of histone-depleted metaphase chromosomes and their treatment with restriction endonuclease Bam HI. Mitotic cells were suspended in 2 ml of the extraction buffer containing 10 mM EDTA - 10 mM tris-HCl, pH 9, 0.1 mM phenylmethylsulfonylfluoride (PMSF), at a DNA concentration of 50 μg/ml. Colcemide was added to a final concentration of 0.1 μg/ml and NP40 upto a 0.5% concentration. The mixture was incubated for 15 min at 0°C. Then the equal volume of 4 M NaCl was added, the solutions were carefully mixed and immediately layered on the surface of a discontinuous gradient consisting of (from bottom to top) (1) 1.5 ml of metrizamide (0.6 M) in the restriction buffer (6 mM $MgCl_2$, 6 mM tris-HCl, pH 7.5, 6 mM β-mercaptoethanol), containing endonuclease Bam HI (100 units/ml); (2) 2 ml of 40% sucrose in the restriction buffer containing 0.1 mM PMSF and Bam HI endonuclease (250-500 units/ml); (3) 5 ml of 35% sucrose in the restriction buffer containing 0.1% NP40 and 0.1 mM PMSF; (4) 7.5 ml of 5% sucrose in the extraction buffer containing 2 M NaCl, 0.1% NP40 and 0.1 mM PMSF. On top of the gradients, 17 ml of the extraction buffer was layered and the tubes were centrifuged in a SW27.2 rotor (Beck-

man) at 22,000 cpm for 40 min at 4°C. In control experiments, endonuclease was not added. After centrifugation 32.5 ml of the solution was carefully removed from top of the gradient. The remaining material containing most of the DNA (at the interphace between the metrizamide layer and 40% sucrose) and restriction endonuclease was incubated for 1 h at 37°C. Thereafter, the phases were carefully mixed and an EDTA solution was added to a final concentration of 10 mM.

Isolation of rapidly sedimenting DNA fraction (DNA presumably bound to the axial structures). After incubation, the material was diluted eight times with 20 mM tris-HCl, pH 7.5, 10 mM EDTA, 0.1 mM PMSF and layered on the top of the second discontinuous gradient consisting of (from the bottom): (1) 1.5 ml of 0.6 M metrizamide, (2) 10 ml of 30% sucrose in 20 mM tris-HCl, pH 7.5, 10 mM EDTA, 0.1 mM PMSF. The centrifugation was performed in a SW27.2 rotor (Beckman) at 22,000 rpm for 45 min at 4°C. Rapidly sedimenting DNA was collected at the border of the metrizamide layer. Slowly sedimenting DNA did not enter 30% sucrose.

DNA was prepared according to /7/ and additionally purified by ultracentrifugation in an equilibrium CsCl density gradient. A solution of CsCl with $\rho = 1.7$ g/cm^3 in 5 mM triethanolamine, 1 mM EDTA, 0.1% sarcosyl, pH 7.0, was used. Ultracentrifugation was performed in a Ti 50 rotor (Beckman) for 48 hr at 40,000 rpm and 18°C or in a Ti 65 rotor (Beckman) for 48 hr at 45,000 rpm and 18°C.

Alternative procedure for the preparation of DNA bound to the axial structures. In several cases, the purified chromosomes were isolated by differential centrifugation after lysis of mitotic cells with NP40. The isolated chromosomes were suspended in the EcoRI restriction buffer (20 mM tris-HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM ß-mercaptoethanol, 0.1 mM PMSF 0.05% NP40, 10% glycerol) and treated with EcoRI restriction endonuclease. The reaction was terminated by a ten-fold dilution of the reaction mixture with an extraction buffer. Dehistonization, nuclease treatment of the DNA which remained bound to the chromosomal axis after dehistonization, and

the second centrifugation were done in the same way, as in the previous case when the chromosomes had not been pretreated with EcoRI. When EcoRI or HindIII restriction endonucleases were used instead of Bam HI for treatment of the dehistonized chromosomes, three lower layers of the first discontinuous gradient were made in an appropriate buffer solution: 40 mM tris-HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol.

DNA labeling with [^{32}P] was done with the aid of nick translation /8/ [α^{32}P] deoxynucleosidetriphosphates with a specific activity of 300 Ci/mmole (Amersham) and DNA polymerase I from Bac.subtilis (Miles Laboratories, Inc.) were used. DNA was purified by chromatography on hydroxyapatite and Sephadex. The specific activity of DNA was 2×10^7 cpm per μg.

Analytical procedures. Renaturation studies were performed either in the presence or in the absence of an excess of total DNA (conditions are specified in legends to figures). The percentage of renatured material was determined with the aid of chromatography on hydroxyapatite /9/.

Electrophoresis of DNA in agarose gels was performed according to /10/.

Prior to gel electrophoresis, proteins were precipitated with 25% TCA, washed with acetone, dried, and dissolved in 1% SDS, 1% β-mercaptoethanol, 10 mM sodium phosphate, pH 7.0. Electrophoresis was performed in 7.5% polyacrylamide gels with 1 M urea as described earlier /11/.

RESULTS

Isolation of DNA fragments attached to the axis of chromosome. The first problem was to obtain high amounts of histone-depleted chromosomes in a dispersed state convenient for the restriction endonuclease treatment. For this purpose, centrifugation through a composite discontinuous sucrose gradient described in the Methods was used. Chromosomes lost histones while moving through a layer containing 2 M NaCl; then, 2 M NaCl was replaced in two steps for a low ionic strength

buffer used for restriction, and finally chromosomes which had lost histones were concentrated in a thin layer at the interface between the sucrose gradient and the metrizamide layer. In a number of experiments, this layer contained endonuclease Bam HI. One can see (Fig. 1a) that the labeled DNA was concentrated mainly on top of the metrizamide layer.

Some part of the [^{3}H] label remaining on top of the gradient depended on the incorporation of [^{3}H]thimidine into RNA in the course of long incubation. Thus, at least 80%, but probably more than 90% of DNA was recovered in the rapidly moving material corresponding to dehistonized chromosomes.

Most proteins were recovered in the top fraction but 15-20% of the labeled protein comigrated with DNA (Fig. 1b). Electrophoretic analysis demonstrated that practically all histones were concentrated in the top fraction (Fig. 3). The

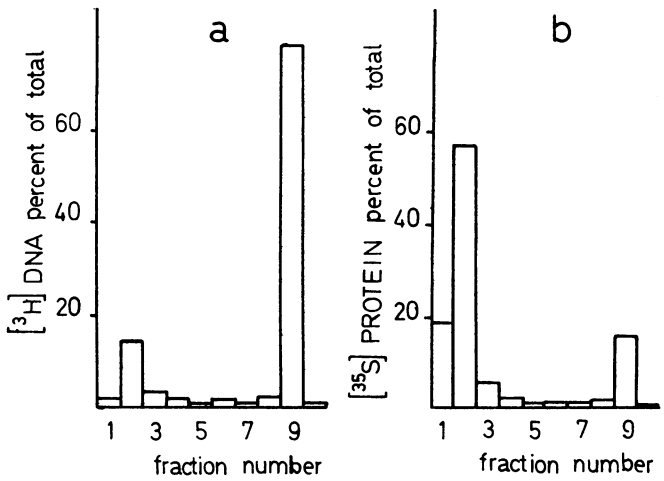

Fig. 1. Distribution of [^{3}H] DNA and [^{35}S] protein after salt extraction of NP40-treated mitotic cells.

A discontinuous gradient was prepared as described in Methods. After centrifugation, 17 ml of the extraction buffer which had been placed on the sample to prevent collapse of the tube during centrifugation were carefully removed. Other portions of gradient were collected into ten fractions from top to bottom. Aliquots for count were taken from each fraction. Fraction N9 contained the fast sedimenting material concentrated on the border of the 0.6 M metrizamide layer.

latter also contained cytoplasmic proteins. The chromosomal fraction contained no visible amounts of histones but some non-histone proteins.

Histone-depleted chromosomes suspended in the narrow region at the interface between the two layers containing endonuclease Bam HI were incubated before the second ultracentrifugation. In the second run, DNA was distributed between the upper layer (DNA cleaved off) and the lower layer containing metrizamide (Fig. 2).

In control experiments where no restriction endonuclease was present, the incubation and other manipulations led to a shift of about 30% of the label into the upper layer (Fig. 2b). The major part of it was still bound to the chromosomal axial structures. After exhaustive treatment with Bam HI endonuclease only 4% of DNA was recovered in the fast sedimenting fraction

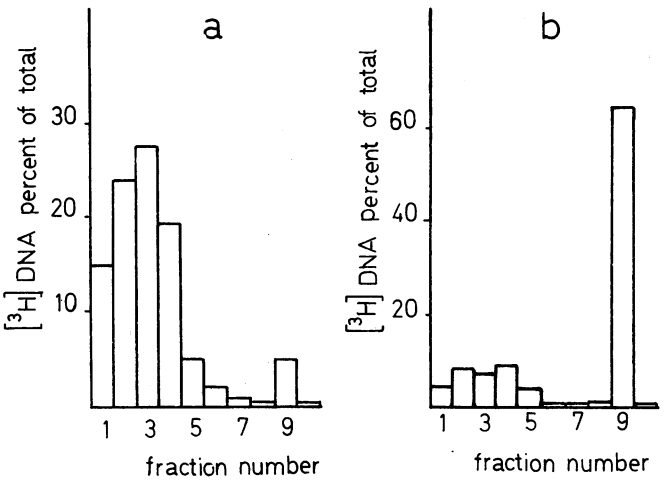

Fig. 2. Distribution of [^{3}H] DNA in the second centrifugation of NaCl extracted chromosomes after nuclease treatment in conditions providing complete digestion (a) and without nuclease treatment (b).

The second discontinuous gradient was prepared as described in Methods. The material was collected into ten fractions from top to bottom after centrifugation. Fraction N9 contained the fast sedimenting material concentrated on the border of the 0.6 M metrizamide layer.

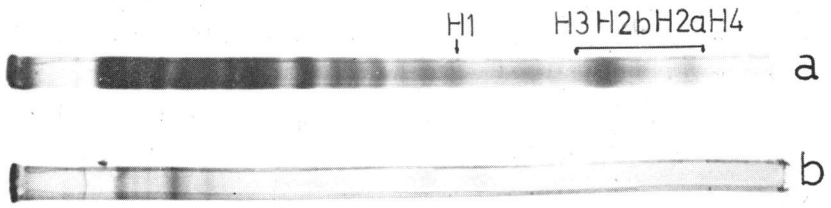

Fig. 3. Electrophoretic analysis of proteins extracted with 2 M NaCl from NP40-treated mitotic cells and proteins sedimenting with DNA after salt extraction.

(a) Proteins extracted with 2 M NaCl.
(b) Proteins from the fast sedimenting fraction obtained after salt extraction (see Methods).

(Fig. 2a). In some experiments, the conditions in which incomplete restriction took place were used and the amount of DNA in the fast sedimenting fraction was higher (about 10%). The bulk of labeled protein was recovered in the fast sedimenting fraction. Thus, the material was not lost due to destruction of the axial structures in the course of incubation with restriction endonuclease.

In experiments where chromosomes had been originally pretreated with EcoRI, much less DNA was found in the fast sedimenting fraction after the first centrifugation (usually about 6%). The second cycle of digestion using the same or another endonuclease gave an approximately five-fold decrease in the amount of the "fast sedimenting DNA". Experiments with EcoRI pretreatment made it possible to use higher amounts of chromosomes for centrifugation due to a reduced viscosity.

The fast sedimenting fractions were collected, and DNA was deproteinized and used in further studies.

Properties of the attached DNA. The process of DNA purification involved the CsCl density gradient ultracentrifugation yielding two DNA components with the buoyant densities of

1.70 and 1.69 g/cm^3. In the case of Bam HI, the lighter peak contained 10-15% of the total DNA. In experiments with EcoRI pretreatment followed by additional EcoRI digestion, the peak was much higher comprising 70% of the total DNA (Fig. 4). The results correlated well with the known resistance of the mouse satellite, to a treatment with EcoRI endonuclease. EcoRI--HindIII treatment reduced the relative content of the lighter component.

In most of the experiments, the light and dense DNA bands were collected separately and labeled additionally by nick--translation.

In some cases, ultracentrifugation in a CsCl density gradient was repeated at this stage. The total mouse DNA was used as an internal marker. One can see (Fig. 4) that the light band of the axis-attached DNA has a buoyant density typical of the mouse satellite (1.69 g/cm^3) while the denser band is similar to the total DNA. Wide distribution of the nick-translated material in a CsCl density gradient depended on the low molecular weight of the nick-translated DNA. DNA fractions additionally labeled by nick-translation were denatured and renatured in the presence of the total cellular DNA used as a reaction driver.

Nick translation made some DNA nondenaturable due to the formation of hairpin-like structures. The latter fraction was removed from samples by hydroxyapatite chromatography performed immediately after denaturation. The decrease of the molecular weight made a certain part of DNA (20-30%) unable to renature even at high C_0t values (10^4 moles x l^{-1} x sec). Therefore, the latter fraction was subtracted and the renaturation at $C_0t = 10^4$ was considered as a 100% renaturation. One can see that the axis bound DNA was strongly enriched in reiterated base sequences comparing to the total DNA (Fig. 5).

In the presence of total DNA, the mouse satellite should renature in the C_0t interval of $5x10^{-4}$ - $5x10^{-2}$ moles x l^{-1}x sec.

One can see (Fig. 5) that the major part of the light peak in the Bam HI treated material corenatured with the satellite fraction of the mouse DNA. Some additional renaturat-

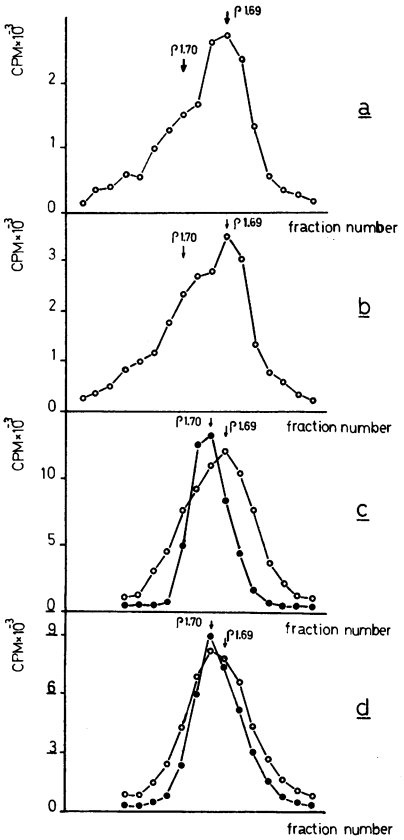

Fig. 4. CsCl equilibrium density centrifugation of the axis-attached DNA.

Equilibrium density centrifugation was performed in a CsCl solution (ρ 1.7 g/cm^3) in 5 mM TEA-HCl, pH 7.0, 1 mM EDTA, 0.1% sarcosyl. Samples were centrifuged in a Beckman Ti50 rotor for 48 hr at 45,000 rpm and 18°C (a,b) or in a Beckman Ti50 rotor for 48 hr at 40,000 rpm and 18°C (c,d).

(a) Axis-attached [^{3}H] DNA after double treatment of chromosomes with EcoRI endonuclease.
(b) Axis-attached [^{3}H] DNA after successive treatment of chromosomes with EcoRI and HindIII endonucleases.
(c,d) The light component and the main band of the nick-translated axis attached DNA obtained in experiments with Bam HI treatment of histone depleted chromatin.

In (c) and (d) , besides the analysed nick translated material (o-o), each tube contained the total DNA from L cells labeled <u>in vivo</u> with [^{3}H] thymidine and sheared to 0.4-0.8 kb (●-●).

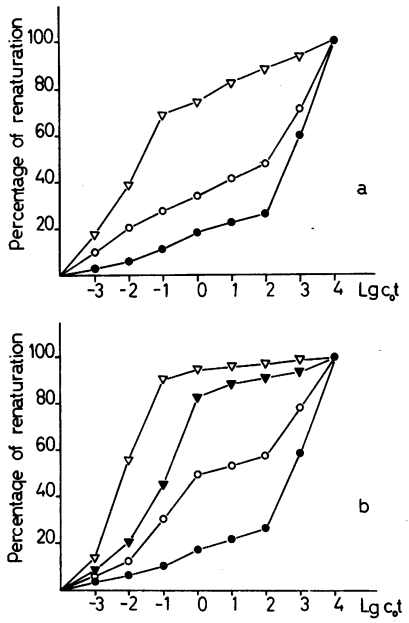

Fig. 5. Renaturation curves for different fractions of the axis-attached DNA.

Renaturation experiments were performed in the presence of an excess of the total DNA from mouse L cells. The driver DNA was sheared to about 0.4 kb. The length of nick-translated DNA chain was from 0.2 to 0.5 kb.

All the renaturation curves were normalized by considering renaturation at $C_0t = 10^4$ as a 100% renaturation. Actually it varied from 70 to 80%.

A. Experiments were performed with DNA of histone-depleted chromosomes treated with Bam HI endonuclease at the point when 10.5% of the material was recovered in the fast sedimenting fraction.

●-●	Total DNA (control experiment).
▼-▽	Light component (~15% of the total axis-attached DNA).
o-o	Major component of the axis-attached DNA.

B. Experiment with the axis-attached DNA from chromosomes pretreated with EcoRI.

●-●	Total DNA (control experiment).
▽-▽	Light component obtained after the second treatment of chromosome with EcoRI endonuclease.
o-o	Dense component from the same experiment.
▼-▼	Dense component from the experiment in which HindIII endonuclease was used for the second treatment.

ion at higher C_0t values may depend on cross-contamination with the material of the major band. In the case of the EcoRI treated material where the light band is higher and therefore its contamination by the denser band is negligible, practically all the DNA from the light band renatures at very low C_0t: half-renaturation takes place at $C_0t = 5 \times 10^{-3}$. Taking into account the density of the light peak which is typical of the satellite (1.69 g/cm^3), one may conclude that it corresponds to the AT-rich mouse satellite fraction. Variations in its content depend on the susceptibility to a certain restriction endonuclease.

The denser band contains the material which also renatures predominantly at low C_0t values. The proportion of the fast renaturing fraction depends on the isolation procedure. It is relatively low in preparations obtained by Bam HI treatment, much higher in EcoRI treated samples, and the highest in the material obtained by EcoRI-HindIII digestion where it reaches 85%. This correlates well with the amount of the material which remained bound to the axis after different treatments. The lower the amount of bound DNA the higher the content of reiterated DNA sequences in the band with buoyant density of 1.70 g/cm^3. Detailed analysis of the C_0t curves shows that the main part of the material renatures in the interval of 10^{-2}--10^{0} moles x l^{-1} x sec, thus representing the most abundant classes of intermediately repeated sequences.

Some part of DNA from the dense band renatures at even lower C_0t values. This may depend either on cross-contamination of the fraction with the satellite, or on the existence of short satellite sequences linked to the other ones, or on the presence of the highly repetitive DNA different from the major satellite component.

Along with the most abundant intermediate repeats, a fraction renaturing in the C_0t interval of 10^{0}-10^{2} is also present. Its content correlates with the concentration of unique sequences. It is higher when more DNA remains bound to the axis.

Several point renaturation experiments were performed also in the absence of the carrier DNA. Renaturation in this

case was driven by the axis-attached DNA itself. The *in vivo* [^{3}H] thymidine label was used for the analysis allowing one to avoid artifacts depending on nick translation of DNA.

In general, the results of such experiments (see Table 1) confirmed those obtained with nick-translated DNA. The DNA was sonicated before analysis and its molecular weight (400-800 base pairs) was somewhat higher than after nick translation. At indefinitely low C_0t, about 15% of attached DNA renatured suggesting the enrichment of the material in palindromes. However, additional control experiments are necessary for a final conclusion. The content of the repeated sequences in the axis-attached DNA was again much higher than in the total DNA. The difference was even more prominent than in the former experiments. This might depend on several factors. First, the molecular weight of the sonicated DNA used (0.4-0.8 kb) was somewhat higher than that of the nick-translated DNA (0.2-0.5 kb). Second, the content of a particular sequence in the fractionated DNA should be higher than in the total one if the sequences were not random distributed. This should shift all the curve to lower C_0t values. The latter seems to be the case as follows from comparing the data presented in Fig. 5a and Table 1.

Finally, the size distribution of the axis-attached DNA after EcoRI endonuclease treatment was determined electrophoretically. The major part of the material was recovered in a slowly moving band corresponding to the EcoRI treated satellite. From a third to a half of the material was heterogeneous and had a lower molecular weight (data not shown). We could detect no specific abundant restriction fragments in electrophoregrams. Apparently, different restriction enzymes should be used for this purpose.

DISCUSSION

The techniques are described which allow one to perform the restriction endonuclease treatment of mitotic chromosomes dehistonyzed according to the Laemmli procedure /2/. The size of DNA remaining bound to the axial structure of a chromosome after exhaustive treatment with endonuclease Bam HI is of 3-4

Table 1

Renaturation of the major band of the axis-attached DNA in the absence of driver total DNA

$C_o t$	Percentage of renaturation	
	axial-attached DNA	total DNA
0	15.2	3
10^{-3}	35.9	8
10^{-1}	47	15
10^{0}	61.5	23

The investigated DNA was sonicated to 0.4–0.8 kb, denatured and renatured in the absence of driver total DNA. The per cent of renaturation for the total DNA which was sonicated, denatured and renatured at the same $C_o t$ values as the investigated DNA is given to facilitate comparison.

In this experiment the axis-attached DNA was obtained with the aid of Bam HI treatment.

kb on the average (data not shown). Considering that about 4% of DNA is still attached to the axis in these conditions, one can estimate the size of DNA between two attachment points as being equal to 70–100 kb. This figure is in agreement with direct measurements of the loop length /2/.

The main conclusion from the presented results is that a DNA·protein fibril is attached to the axial structure of a chromosome non randomly. As follows from renaturation experiments, sequences closely adjacent to attachment points are enriched in reiterated DNA sequences including the satellite and the most abundant classes of intermediately repeated DNA. These sequences seem to be associated most closely with the axial structure. On the other hand, they are probably linked to less abundant and unique DNA sequences. Experiments with renaturation in the absence of an external driver suggest that the latter represents a certain subset of the total fraction of intermediate repeats. They also suggest the enrichment

of the axis-attached DNA with palindromes. However, both statements require further checking.

It has been suggested that large chromosome DNA·protein loops correspond to independent structural-functional elements of the genome /4-6/. If this is true, then using the attachment sequences as markers for the boundaries of such genome elements, one can analyze the structural organization of the latter. This work is being in progress in our laboratory.

In the course of preparing this paper the authors learned that U.K.Laemmli, et al. (personal communication) obtained data showing the existence of unique simple sequence cut out by EcoRI endonuclease in the axis-attached DNA fraction from HeLa cells. In the case of mouse chromosomes we have been yet unable to find a specific abundant restriction fragment.

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