Intranuclear Anchoring of Repetitive DNA Sequences: Centromeres, Telomeres, and Ribosomal DNA

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Abstract. Centromeres, telomeres, and ribosomal gene clusters consist of repetitive DNA sequences. To assess their contributions to the spatial organization of the interphase genome, their interactions with the nucleoskeleton were examined in quiescent and activated human lymphocytes. The nucleoskeletons were prepared using "physiological" conditions. The resulting structures were probed for specific DNA sequences of centromeres, telomeres, and ribosomal genes by in situ hybridization; the electroeluted DNA fractions were examined by blot hybridization. In both nonstimulated and stimulated lymphocytes, centromeric alpha-satellite repeats were almost exclusively found in the eluted fraction, while telomeric sequences remained attached to the nucleoskeleton. Ribosomal genes showed a transcription-dependent attachment pattern: in unstimulated lymphocytes, transcriptionally inactive ribosomal genes located outside the nucleolus were eluted completely. When comparing transcription unit and intergenic spacer, significantly more of the intergenic spacer was removed. In activated lymphocytes, considerable but similar amounts of both rDNA fragments were eluted. The results demonstrate that: (a) the various repetitive DNA sequences differ significantly in their intranuclear anchoring, (b) telomeric rather than centromeric DNA sequences form stable attachments to the nucleoskeleton, and (c) different attachment mechanisms might be responsible for the interaction of ribosomal genes with the nucleoskeleton.

Key words: human lymphocytes • interphase nuclei • nucleoskeleton • nuclear matrix • fluorescence in situ hybridization

R EPETITIVE DNA sequences in the human genome are numerous and diverse in their base composition. Several of them are clustered and possess clearly defined positions on chromosomes. The most prominent are the alpha-satellite repeats of centromeres, the telomeric repeats, and the clusters of ribosomal gene repeats. They have different functions as outlined in the following.

Alpha-satellite repeats are the main component of the centromeres and pericentromeric regions of all human chromosomes (Mitchell et al., 1992; Murphy and Karpen, 1998). Centromeric DNA together with the associated proteins (CENPs; e.g., Haaf and Ward, 1994; He et al., 1998) are the key structures where the mitotic spindle anchors. In most eucaryotes, the mitotic spindle is essential to accomplish an equal distribution of the genetic material into the two daughter cells (Rattner, 1991; Wood et al., 1997). These DNA regions do not house active genes (Brown et al., 1997).

Telomeres generally consist of a highly repetitive hexanucleotide sequence of TTAGGG, although two other telomeric repeats have also been described (Allshire et al., 1989). The average length of these repeats are species and cell type specific (Lejnine et al., 1995; Lansdorp et al., 1996). Furthermore, since some telomeric DNA is lost in every round of DNA replication, the length of telomeres is indicative of the number of cell cycles a normal somatic cell has gone through. In cycling cells, it is therefore an indicator of cell age and may have an important role in the process of aging of individuals (Vaziri et al., 1993; Allsopp and Harley, 1995; Allsopp et al., 1995; Henderson et al., 1996; Notaro et al., 1997; Weng et al., 1997; De Boer and Noest, 1998). The telomeres are associated with a number of specific proteins (Zakian, 1995; Gotta et al., 1996; Barinaga, 1997; Cockell et al., 1998). A disturbance of the "telomeric complex" can result in end-to-end fusion of chromosomes (Hawley, 1997; Mondello et al., 1997; Slijepcevic et al., 1997; van Steensel et al., 1998) and/or in malignant transformation of cells (Zakian, 1995; Blasco et al., 1997).

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The telomeres are considered to be transcriptionally inactive.

Human ribosomal genes are organized in repeats at the secondary constriction of the five acrocentric chromosomes. In contrast to the centromeres and telomeres, this part of the genome can be transcriptionally highly active if cell proliferation and protein synthesis are high (Schwarzacher and Wachtler, 1993; Raska, 1995; Shaw and Jordan, 1995; Sirri et al., 1995; Pederson, 1998).

In addition to the above mentioned functions, it has been speculated that centromeres and telomeres have an organizing function in the spatial genome arrangement within the interphase nucleus (Haaf and Schmid, 1991; He and Brinkley, 1996). More than 100 yr ago, Rabl (1885) proposed a model of polarized orientation of interphase chromosomes with the centromeres at one side of the nucleus, and with the telomeres facing the other side. Since then, numerous studies on interphase chromosome arrangement have been performed and there is no doubt that the chromosomes in the interphase nucleus are not randomly distributed (e.g., Hilliker and Appels, 1989; Cremer et al., 1993; Lamond and Earnshaw, 1998). An ordered, nonrandom arrangement is especially evident for the centromeres, which tend to associate with the nuclear membrane and with the nucleolus (Manuelidis, 1984; Billia and De Boni, 1991; Ochs and Press, 1992). The nonrandom distribution raises the question how this order is maintained and whether there exist structures that may serve as basic anchoring points that regulate the positioning of individual chromosomes and subchromosomal domains.

An underlying fibrillar structure such as the nucleoskeleton, or the nuclear matrix, is probably the best candidate for such a positioning structure (Berezney and Coffey, 1975, 1977; Mirkovitch et al., 1984; Hozák, 1996; Nickerson et al., 1997). The fibrillar nature of the nucleoskeleton was clearly demonstrated, and some of the principal components of the nucleoskeleton have been identified in situ—such as heteronuclear RNPs, the nuclear mitotic apparatus protein, and intermediate filaments; e.g., lamins (Jackson and Cook, 1988; He et al., 1990; Nakayasu and Berezney, 1991; Hozák et al., 1995; Mancini et al., 1996; Mattern et al., 1997).

The question about the mechanisms of the interactions between the nucleoskeleton on one side, and the DNA or chromosomes on the other, has been addressed by many authors, sometimes with controversial results. Nevertheless, the postulated interactions can be divided into two principally different groups: first, interactions have been described between the nucleoskeleton and specific DNA sequences (MARs, SARs) that have rather permanent character and are responsible for chromatin loop formation (Getzenberg et al., 1991; Ivanchenko and Avramova, 1992; Boulikas, 1995; Gonzales and Sylvester, 1995; Razin, 1997). Second, interactions between functional DNA/protein complexes and the nucleoskeleton have been found, such as in transcription or replication foci. These attachments are thought to be rather transient than permanent (Jackson and Cook, 1985b, 1986; Hozák et al., 1993; Hyrien et al., 1997; Jackson, 1997; Stein et al., 1998).

As outlined above, the centromeres, telomeres, and ribosomal gene repeats have very different functions; in ad-

dition, they all might contribute to or influence the spatial organization of the genome in different ways. These contributions are poorly understood, and the mechanisms of the interactions have been identified only in a few cases.

Telomeric repeats have been shown to be matrix-associated in cultured cells (de Lange, 1992; Ludérus et al., 1996). Conflicting results exist concerning the presence of attachment sites within the centromeric alpha-satellite repeats (Jackson et al., 1996; Strissel et al., 1996; Craig et al., 1997). For the ribosomal gene repeat, diverging observations were reported with respect to intranuclear attachment. Several studies describe an interaction with the nuclear matrix only for the intergenic spacer (Smith and Rothblum, 1987; Stephanova et al., 1993). Others, however, have observed rDNA/nuclear matrix attachments throughout the entire ribosomal gene repeat (Keppel, 1986; Maric and Hyrien, 1998). Using gentle techniques for nucleoskeleton preparation (Jackson et al., 1988), associations of the transcription unit with the nucleoskeleton were observed in HeLa cells, whereas the intergenic spacer was attached to the nucleoskeleton only rarely (Dickinson et al., 1990; Weipoltshammer et al., 1996b).

The terminology used above illustrates the methodological difficulties of mapping the attachment sites: "nuclear matrix" protocols involve high-salt extraction, "nuclear scaffold" preparations involve the use of lithium diiodosalicylate, whereas "nucleoskeleton" preparations are generated with a more gentle, "physiological" method of chromatin removal. The many different approaches and cell models used make it very difficult to compare data on chromatin attachment. In addition, it is generally accepted that changes in the expression level of a gene can change its intranuclear attachment; however, only a few systematic studies exist on that issue (Craig et al., 1997; for a review see Stein et al., 1998). Throughout this paper, we use the terminology matching the extraction protocols applied.

In this paper, we compare the patterns of attachment of three functionally different repetitive genome elements, centromeres, telomeres, and ribosomal genes, to the nucleoskeleton, using a gentle and controlled method of chromatin extraction. Human lymphocytes before and after growth stimulation were used to compare nuclear attachment in cells with low and high levels of nuclear transcription. By using in situ evaluation, we took a different approach than in previous studies. This in situ method allowed us to investigate qualitative and quantitative signal characteristics at the single-cell level. We combined the in situ analysis of the DNA sequences remaining in the nucleus after chromatin removal with an analysis of the extracted DNA fraction by Southern blot hybridization. Double in situ hybridization experiments were performed to investigate attachment characteristics of the repetitive genome elements in single cells: telomeres plus ribosomal genes or centromeres plus ribosomal genes were detected simultaneously in control and extracted cells. The amount of DNA remaining in the nucleus after electroelution was quantified by densitometrically evaluating the signals after in situ hybridization. This method is complementary to procedures using biochemical fractionation of isolated DNA before and after chromatin depletion (e.g., Jackson et al., 1996). The method of quantification we used enables

us to evaluate the degree of nucleoskeleton anchoring and at the same time to visualize attached DNA fragments at the single cell level.

The aim of this study is to investigate whether: (a) the various repetitive DNA sequences differ in their intranuclear anchoring, (b) transcriptional activation of cells results in a change of attachment characteristics of the three repetitive DNA sequences studied, and (c) the activation of nucleolar transcription is connected with a spatial rearrangement of specific rDNA elements relative to the nucleoskeleton.

Materials and Methods

Cells

Human lymphocytes were separated according to standard procedures. The separated lymphocytes were washed twice in PBS and embedded in low temperature-melting agarose (type VII; Sigma Chemical Co.) according to the protocol of Weipoltshammer et al. (1996b). One part of the embedded cells was subjected to the nucleoskeleton preparation procedure. The other part was incubated in RPMI medium (supplemented with 20% fetal calf serum and phytohemagglutinin; Abbott Laboratories) at 37°C for 72 h. Stimulated lymphocytes were then washed twice in PBS and subsequently nucleoskeletons were produced.

Preparation of the Nucleoskeletons

All solutions were prepared with diethylpyrocarbonate (DEPC)-treated distilled water (0.1%, inactivated) to minimize RNase content. Agarose-embedded cells were transferred into 0.1 M Soerensen buffer (SB; 70 mM Na_2HPO_4, 30 mM KH_2PO_4, pH 7.4), and then incubated in 0.05 M SB for 5 min at 37°C to disperse the granular component of nucleoli (Hozák et al., 1990, 1992). Afterwards, cells were permeabilized in two changes of lysis buffer (70% PB diluted with DEPC-water, supplemented with 0.2% Triton X-100, 0.1 mM PMSF, and 2.5 U/ml RNase inhibitor; Amersham International), 5 min each on ice, and washed in "physiological buffer" (PB; 70 mM K-acetate, 30 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM Na_2ATP, 1 mM DTT, 0.1 mM PMSF, pH 7.4 adjusted with KH_2PO_4, 2.5 U/ml RNase inhibitor; Amersham International) 4×10 min on ice. Chromatin in permeabilized cells was cut using 340 U/ml HaeIII (Roche Laboratories) and 1,650 U/ml EcoRI (Roche Laboratories) in the presence of 25 U/ml RNase inhibitor (Amersham International); the reaction was carried out in a 3-ml vol bead suspension in PB for 20 min at 33°C. For the telomeric repeat, we cross-checked our results by cutting one sample of stimulated lymphocytes with RsaI (1,000 U/ml; Sigma Chemical Co.) and HinfI (1,000 U/ml; Roche Laboratories) that cut within the subtelomeric DNA sequences (Vaziri et al., 1993). No difference between the two samples was found. Subsequently, the bead suspension was introduced into the slots of an agarose gel (0.8% in tetraethylammonium [TAE] buffer, supplemented with 2.5 U/ml RNase inhibitor). Electrophoresis was run for 30 min at 20 V, and then for 2 h and 30 min at 35 V. For electrophoresis, PB diluted with DEPC-water to 70% and containing 0.1 mM PMSF and 0.5 U/ml RNase inhibitor was used as a running buffer. Cells were retrieved from the slots and fixed in 4% formaldehyde in PBS for 30 min.

Control samples were fixed with 4% formaldehyde in PBS for 30 min without any enzymatic digestion of DNA. All samples were washed twice in PBS, dehydrated in graded series of methanol, and stored in 100% methanol at −20°C for further analysis.

After recovering agarose beads from the gel slots, the gel was treated with RNAse A (0.5 μg/ml, 1 h, 30°C), proteinase K (5 μg/ml, 1 h, 20°C), and stained with ethidium bromide as described (Jackson and Cook, 1986). The gel was photographed and blotted onto a Nylon membrane (Roche Laboratories) for hybridization.

As an internal control, DNA was isolated from three fractions of cells by phenol extraction: (a) before cutting with restriction enzymes, (b) after cutting with EcoRI and HaeIII before electrophoresis, and (c) after electrophoresis. With these three samples of isolated DNA, gel electrophoresis (1.7% agarose in TAE buffer) was performed.

DNA In Situ Hybridization

The agarose-embedded cells were spread over aminoalkylsilane-coated slides (Polysciences Inc.) and immobilized by incubating the slides at 65°C for 3 d. Fluorescence DNA in situ hybridization on nucleoskeleton preparations and control cells was performed according to Wachtler et al. (1991). Instead of proteinase K, 0.1% pepsin in 0.01 N HCl (pH < 2) was used (Dirks et al., 1989). To optimize hybridization conditions for the embedded cells, the formamide content of the hybridization mix was reduced to 30%. Sample and probe were denatured simultaneously on the slide for 10 min at 95°C. The hybridization took place at 37°C overnight in a moist chamber. Stringency washes were carried out at 42°C with $0.6\times$ standard sodium citrate containing 20% formamide (three changes, 10 min each).

For the detection of telomeres and centromeres, commercially available probes were used: the digoxigenin-labeled "all human telomere DNA-probe" consisting of the TTAGGG repeat (Vysis), and the biotin-labeled "all human centromere probe" containing centromeric alpha-satellite sequences (Vysis). Alternatively, we used a Cy3-labeled PNA-telomere probe (DAKO SA) that has a higher sensitivity but a lower staining intensity. The probes for the detection of the ribosomal gene repeat were the EcoRI A fragment (part of the transcribed unit; stretching from the 3′ end of 18S to the 3′ end of 28S subunit, thereby including both internal transcribed spacers and the 5.8S subunit of the ribosomal gene; the A fragment measures 7.1 kb), and the HindIII D_{HH} fragment (located within the noncoding intergenic spacer; it is part of the EcoRI-defined D fragment; the D_{HH} fragment measures 9 kb). These probes were kindly donated by Prof. James Sylvester. They were labeled by nick-translation (nick-translation kit; Roche Laboratories) with either digoxigenin or biotin. For simultaneous detection of telomeres and rDNA, the digoxigenin-labeled "all human telomere probe" in combination with either A or D_{HH} fragment, both biotin-labeled, was used. Simultaneous detection of centromeres and rDNA was carried out with the biotin-labeled "all human centromere" probe plus the digoxigenin-labeled rDNA fragments. Detection of DNA probes was performed with commercially available antibodies against digoxigenin (rhodamine conjugated), or using FITC-conjugated avidin in the biotin/avidin detection system (Pinkel et al., 1986) as in Weipoltshammer et al. (1996a).

Southern Blots

After electroelution, the agarose gel containing the eluted fraction of chromatin was blotted onto a Nylon membrane using 0.4 N NaOH overnight. DNA was fixed on the membrane by baking for 30 min at 110°C. The total extracted DNA was detected by hybridization with human genomic DNA as a probe (Sigma Chemical Co.). Specific sequences (telomeres, centromeres, and the ribosomal gene segments A and D_{HH}) were detected with the same probes used for in situ hybridization. All DNA probes were digoxigenin-labeled, and then detected with antidigoxigenin antibodies conjugated with alkaline phosphatase (Roche Laboratories); NBT/X-phosphate solution (Roche Laboratories) was used as a substrate.

Imaging and Evaluation In Situ

Optical sections of nuclei were recorded with a confocal laser scanning microscope (LSM 410; Carl Zeiss, Inc.). The nuclei displayed in Figs. 2–5 are projections of stacks of optical sections.

Control and eluted cells were densitometrically evaluated for several criteria. For all in situ evaluations, confocal series of nuclei were gained under identical conditions (pinhole, resolution, z distance, gain, contrast, etc.).

First of all, the elution efficiency for DNA was checked. Control and eluted cells of unstimulated and stimulated lymphocytes were stained with quinacrin mustard. Samples were recorded and densitometric quantification of the sections was carried out with KS400 software (Kontron). The samples were segmented with a fixed threshold. The total signal density per nucleus was calculated from the optical sections and the mean signal density per nucleus was determined for each sample. Elution efficiency was expressed as the relation of mean signal density per nucleus of eluted cells to the control cells.

The same method was used to compare hybridization signals between control and eluted cells. For all sequences hybridized (telomeric repeat, alpha-satellite repeat, and A and D_{HH} fragments) the relations of mean signal density per nucleus of eluted cells to the corresponding control cells were calculated.

Imaging and Evaluation of Blot Hybridizations

The hybridized membranes were digitized with a flat-bed scanner. The

signal was densitometrically evaluated and normalized against the membrane background with KS400 software. Linearity of the detection system was checked with a dilution series of the alkaline phosphatase-labeled antibody. For comparison of A and D_{HH} fragments, the densities of the hybridization signals were measured and given as relative value. To correct for differences in probe characteristics, a dilution series of total human DNA was blotted and hybridized with the A as well as the D_{HH} fragment. The relation of the signal intensity of A and D_{HH} fragments was the same for all DNA concentrations. The obtained ratio of signal intensity of A versus D_{HH} fragment was used to normalize the hybridization signal of the electroeluted DNA fraction. The amount of extracted A and D_{HH} fragments was compared within the sample of unstimulated lymphocytes and within the sample of stimulated lymphocytes densitometrically. The extracted total DNA was visualized on blots of the same elution experiments after hybridization with a total genomic probe.

Results

Nuclear Extraction

Nuclear morphology after the electroelution was well preserved as checked at the electron microscopic level (data not shown). In both unstimulated and stimulated lymphocytes, the DNA was generally extracted in equal amounts (>80% of DNA) as assessed with densitometric evaluation after quinacrin mustard staining (Table I). The amount of extracted DNA is in line with data from the literature (compare Dickinson et al., 1990). The permeabilization conditions used in our experiments represent a good balance between preservation of nuclear morphology and a high yield of DNA extracted.

The reliability of the electroelution procedure was checked on the DNA level at several points. DNA was isolated from the agarose-embedded cells after restriction enzyme cutting (EcoRI, HaeIII) and directly after electroelution. Both samples were subjected to electrophoresis (Fig. 1 a). Total DNA after restriction enzyme cutting showed a DNA smear plus nucleosomal bands in the lower weight range (lane 2). The DNA fraction remaining in the nucleus after electroelution also shows a smear, the nucleosomal

Figure 1. Gel electrophoresis from different steps of nucleoskeleton preparations from stimulated human lymphocytes. (a) DNA was purified from agarose-embedded cells without cutting with enzymes (controls, 1), after cutting with *EcoRI* and *HaeIII* (2), and after cutting and removal of chromatin (3). Note the presence of nucleosomal bands in lane 2. Samples of DNA (1 μg in 1 and 2 and 0.5 μg in 3) were subjected to electrophoresis on a 1.7% agarose gel (M1: λ/EcoRI, HindIII; M2: pUCBM21/HpaII, DraI, HindIII). (b) Extracted fraction of chromatin after gel electrophoresis of cells cut with EcoRI and HaeIII. The two lanes displayed were loaded with roughly equal numbers of beads. Electrophoresis was performed on a 0.8% agarose gel that was subsequently treated with RNAse A and proteinase K (see Materials and Methods) before staining with ethidium bromide. (c) DNA hybridization of the blotted extracted fraction of chromatin using a human total genomic probe.

bands are no longer found (lane 3). This pattern is well in line with published pictures of extracted DNA from nucleoskeleton preparations (e.g., Jackson et al., 1996).

The extraction of chromatin from nuclei results in a smear in the agarose gel (Fig. 1 b). Nucleosomal bands are not clearly visible and are likely to be obscured by other extracted charged molecules (compare Jackson and Cook, 1986). The corresponding blot hybridization with a total genomic probe is shown in Fig. 1 c. A prominent portion of DNA from eluted chromatin is present in the high–molecular weight range. In this respect, it should be added that it has been shown that chromatin containing DNA fragments larger than 125 kb can be removed from nuclei during preparation of nucleoskeletons (Jackson and Cook, 1985a).

Centromeres

In metaphase cells, the "all human centromere DNA-probe" hybridized to all centromeres, resulting in bright foci, including the pericentromeric regions (data not shown). In the agarose-embedded control interphase nuclei, the hybridization signal was highly clustered (Fig. 2 a) and located preferentially at the nuclear periphery and around the nucleolus in unstimulated as well as stimulated lymphocytes (see Fig. 5 a). In nucleoskeleton prepara-

Table I. In Situ Quantification of Chromatin Depletion

	Unstimulated lymphocytes		Stimulated lymphocytes	
	Control	Eluted	Control	Eluted
	%	%	%	%
Total DNA	100 ± 19.9	18.3 ± 5.3	100 ± 38.7	19.9 ± 9.4
Centromeric DNA	100 ± 20.7	1.7 ± 0.5	100 ± 33.1	1.8 ± 0.9
Telomeric DNA	100 ± 20.6	92.4 ± 1.7	100 ± 30.6	87.7 ± 13.8
rDNA				
A fragment	100 ± 60.4	11.1 ± 6.8	100 ± 42.7	9.5 ± 5.4
D_{HH} fragment	100 ± 44.9	6.1 ± 6.3*	100 ± 39.3	9.8 ± 3.8

Summary of the densitometric evaluation of control and electroeluted cells in situ. Total DNA was stained with quinacrin mustard, centromeric and telomeric repeats and rDNA fragments were labeled by fluorescence in situ hybridization. Cells were recorded and mean values of signal intensities in control and corresponding electroeluted samples were evaluated as described in Materials and Methods. The mean values are displayed as a percentage of signal intensities in electroeluted and control cells (set to 100%). Standard deviations are given as a percentage of the mean value of the control sample. Student's t test was performed for all experiments. Differences in signal intensity between control and eluted samples were highly significant ($P < 0.001$), with the exception of samples probed for telomeres (NS). When comparing unstimulated and stimulated lymphocytes, no significant differences in elution efficiency were found except for the D_{HH} fragment of rDNA (*$P < 0.05$). In addition, the D_{HH} fragment is extracted to a greater amount (*$P < 0.05$) than the A fragment in unstimulated lymphocytes, whereas no significant difference can be found in stimulated lymphocytes. For each sample, at least 20 randomly chosen cells were evaluated.
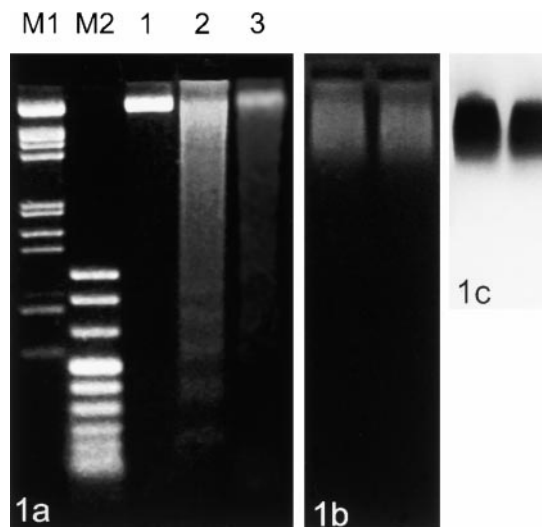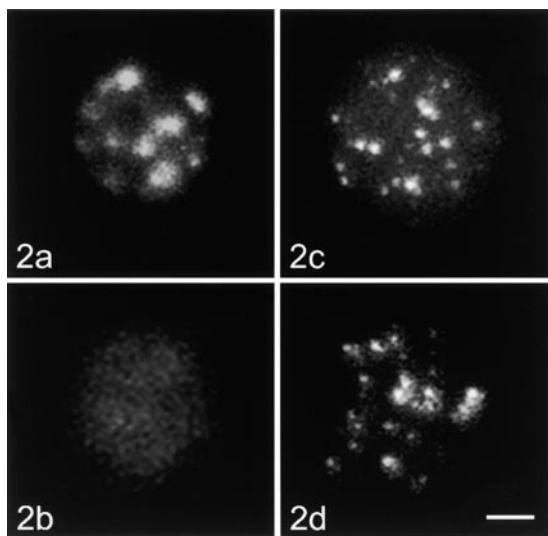
Figure 2. Unstimulated human lymphocytes, control cells, and nucleoskeleton preparations hybridized with the centromeric alpha-satellite probe and the telomeric repeat probe. Nucleoskeleton preparations were produced by permeabilizing the agarose-encapsulated cells with 0.2% Triton X-100 and cutting of DNA with the restriction enzymes EcoRI and HaeIII. Afterwards, the cells were placed into the slots of an agarose gel, electroeluted, and used for in situ hybridization. All figures are projections of stacks of optical sections. (a) Control cell, centromeric probe; (b) nucleoskeleton preparation, centromeric probe; (c), control cell, telomeric probe; (d) nucleoskeleton preparation, telomeric probe. Bar, 2 μm.

tions, the hybridization signal patterns were dramatically changed. The overall signal intensity was reduced and distinct signal clusters were no longer clearly visible in both unstimulated and stimulated lymphocytes (see Figs. 2 b and 5 b). Quantification revealed that <2% of signal specific for centromeric DNA remained in the nucleus after electroelution (Table I).

The blot hybridization of the extracted chromatin fraction (i.e., the unattached chromatin fragments) proved that a considerable amount of centromeric alpha-satellite sequences was removed during the extraction procedure regardless of the overall transcriptional activity of the cells used for elution (see Fig. 6 a).

Telomeres

In chromosome spreads, the all human telomere DNA-probe produced bright dots at the chromosomal ends (data not shown). In interphase control nuclei, the hybridization signal was visible as bright foci distributed throughout the entire volume of the nucleus, in both unstimulated (Fig. 2 c) and stimulated (see Fig. 5 c) cells. Hybridization with the PNA-telomere probe generated identical results (data not shown). In nucleoskeleton preparations of unstimulated and stimulated cells, no apparent differences in signal intensity and distribution were found when compared with the control cells (see Figs. 2 d and 5 d). Densitometric evaluation showed that ~90% of telomeric DNA remained in the nucleus after nuclear extraction (Table I).

Southern blot hybridization of extracted chromatin frag-

ments with the all human telomere probe produced only a very faint signal (see Fig. 6 b). This faint signal is most likely attributed to extra-telomeric TTAGGG repeats (de Lange, 1992; Azzalin et al., 1997).

Ribosomal Genes

In metaphase spreads, both rDNA probes marked the nucleolus organizer regions on all five acrocentric chromosome pairs (data not shown). In interphase control nuclei, both probes hybridized to the nucleolus. In unstimulated cells, this signal was usually roundish or ring-shaped (Fig. 3, a and c). In addition to the typical nucleolar signal, one to several small dot-like signals were observed in ~50% of the unstimulated lymphocytes. These dots correspond to the silent ribosomal gene clusters located outside of the active nucleolus (Wachtler et al., 1986). In stimulated lymphocytes, the nucleolar signal was much more prominent and extended; the extra-nucleolar signals were absent (Fig. 4, a and c).

When in situ hybridization was performed on chromatin-depleted nuclei of unstimulated lymphocytes, the remaining nucleolar signal consisted of one or occasionally a cluster of several dots for both rDNA fragments. The small extranucleolar signals representing silent gene repeats were completely removed from nuclei. An overall loss of signal intensity was seen for the A as well as the D_{HH} fragment (Fig. 3, b and d). However, densitometric quantification revealed that significantly more (factor 1.8) of the A than the D_{HH} fragment remained in the nucleus after electroelution (Table I). Some of the cells had no hybridization signals at all; the percentage of such cells was different for the two rDNA fragments (34.6% for the D_{HH} fragment and 14.6% for the A fragment).
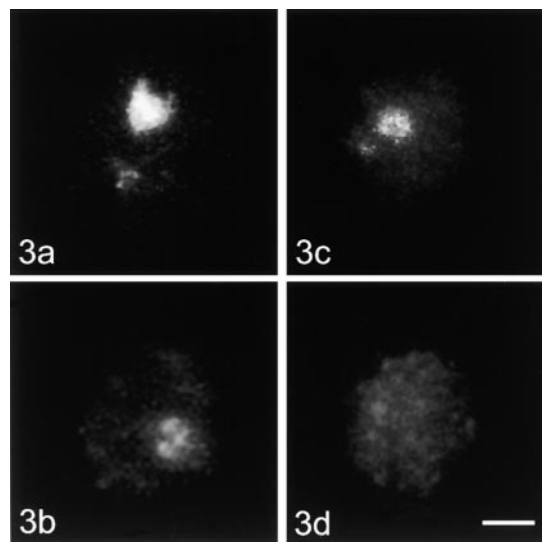

Figure 3. Unstimulated human lymphocytes, control cells, and nucleoskeleton preparations hybridized with the A (part of the transcribed unit) and D_{HH} (part of the intergenic spacer) fragments of the ribosomal DNA. (a) Control cell, A fragment; (b) nucleoskeleton preparation, A fragment; (c) control cell, D_{HH} fragment; (d) nucleoskeleton preparation, D_{HH} fragment. Bar, 2 μm.
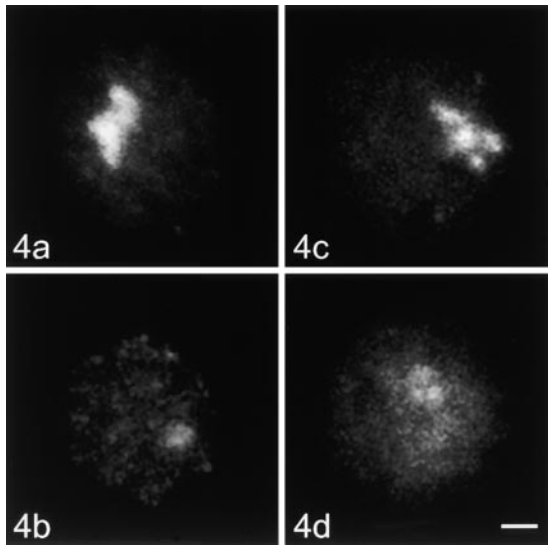
Figure 4. Stimulated human lymphocytes, control cells, and nucleoskeleton preparations hybridized with the A (part of the transcribed unit) and D_{HH} (part of the intergenic spacer) fragments of the ribosomal DNA. (a) Control cell, A fragment; (b) nucleoskeleton preparation, A fragment; (c) control cell, D_{HH} fragment; (d) nucleoskeleton preparation, D_{HH} fragment. Bar, 2 μm.

Figure 5. Stimulated human lymphocytes, control cells, and nucleoskeleton preparations hybridized simultaneously with centromeric alpha-satellite probe and ribosomal DNA probe, or with telomeric repeat probe and ribosomal DNA probe. (a) Control cell, centromeric probe detected with FITC-labeled avidin and A fragment detected with rhodamine-labeled antibodies; (b) nucleoskeleton preparation, hybridization protocol as in a; (c) control cell, D_{HH} fragment detected with FITC-labeled avidin, telomeric probe detected with rhodamine-labeled antibodies; (d) nucleoskeleton preparation, hybridization protocol as in c. Bar, 2 μm.

In the case of in situ hybridizations to stimulated lymphocytes after chromatin depletion, the signal consisted of one to several dots for both probes (Fig. 4, b and d). The signal intensity was also considerably lower than in control cells. In this case, however, densitometry showed that equal amounts of both fragments were removed from nuclei (Table I). The percentage of cells lacking any signal was practically identical for both probes hybridized (22.1% for A fragment and 23.4% for D_{HH} fragment). The results of double in situ hybridization with telomeric and rDNA probes (or with centromeric and rDNA probes) proved that the differences in attachments of the various repetitive genome elements were reproducible on a single cell level (Fig. 5, a–d); thus, these differences cannot result from cell-to-cell variations in the efficiency of chromatin depletion.

The eluted chromatin fragments were blotted and hybridized with A and D_{HH} probes. The hybridization intensities were semiquantitatively compared under normalized conditions. In unstimulated lymphocytes, significantly more of the D_{HH} than the A fragment was removed from the cells by the extraction procedure (relative gray values: A:D_{HH} = 1.62:1; a lower value means higher signal intensity; Fig. 6 c). In stimulated lymphocytes, however, no difference between the amount of eluted A and D_{HH} fragments was detected (relative gray values: A:D_{HH} = 0.97:1; Fig. 6 d). The fraction of stimulated lymphocytes contained ~2% of mitotic cells with potentially different parameters for detaching chromatin fragments, as indicated by Gerdes et al. (1994) and Craig et al. (1997). The low abundance of mitotic cells allows us to neglect the possible effect on the quantified hybridization values.
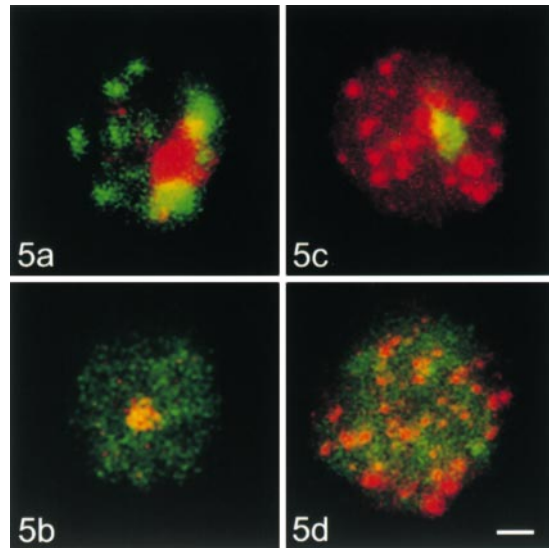
Discussion

The Approach

The principal aim of this paper was to compare the attachment of three functionally different repetitive genome elements: centromeres, telomeres, and ribosomal genes to the nucleoskeleton. The experimental model, human lymphocytes during their metabolic activation, was chosen for two reasons. Firstly, the model provides an excellent system to study possible changes in chromatin/nucleoskeleton attachment during transcriptional activation. Secondly, the model allows us to use native cells without the possible effects of long-term cultivation. Our approach is based on a gentle and controlled removal of unattached chromatin fragments from permeabilized cells (Jackson et al., 1988), followed by a combination of detection of the DNA sequences remaining in the nucleus by in situ hybridization, and that of the extracted DNA fraction by Southern blot hybridization. The extraction method used is known to preserve not only the basic morphological characteristics of the nucleoskeleton, but also the synthetic activities of cell nuclei (Jackson and Cook, 1985b, 1986; Hozák et al., 1993). The in situ approach allowed us to investigate signal intensity as well as signal distribution at the single cell level. Double in situ hybridization experiments served as a reliable control to describe attachment characteristics of the repetitive genome elements simultaneously in a single cell, thus eliminating possible errors when comparing parallel experiments.
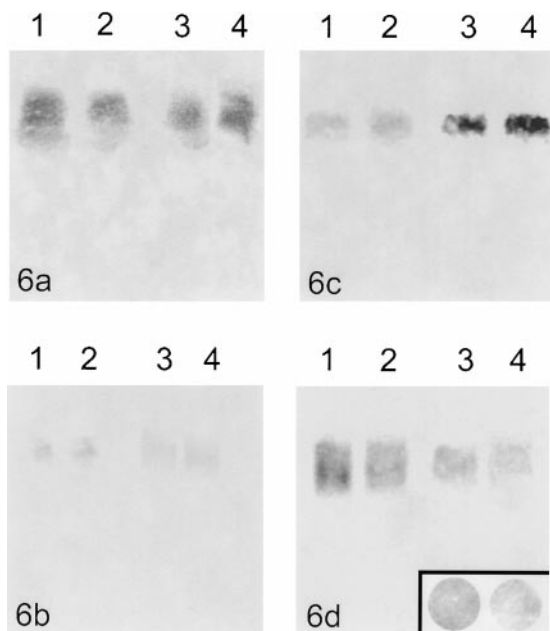
Figure 6. Blot hybridization of chromatin electroeluted from beads. (a) Hybridization with the centromeric alpha-satellite probe; (1 and 2) unstimulated lymphocytes, (3 and 4) stimulated lymphocytes. (b) Hybridization with the telomeric probe; (1 and 2) unstimulated lymphocytes, (3 and 4) stimulated lymphocytes. The very faint signal represents most likely nonattached extratelomeric TTAGGG repeats. (c) Unstimulated lymphocytes; (1 and 2) hybridization with the A fragment (part of the transcribed unit of rDNA), (3 and 4) hybridization with the D_{HH} fragment (part of the intergenic spacer of rDNA). (d) Stimulated lymphocytes; (1 and 2) hybridization with the A fragment, (3 and 4) hybridization with the D_{HH} fragment. The insert shows dot blots of equal amounts of human placenta-DNA hybridized with the A (left) and D_{HH} (right) fragments. To be able to compare the amount of eluted A and D_{HH} fragment in unstimulated as well as stimulated lymphocytes, the signal intensities in the blots c and d were normalized against these dot blots. This results in relative values for signal intensities A:D_{HH} = 1.62:1 in unstimulated lymphocytes and A:D_{HH} = 0.97:1 in stimulated lymphocytes.

Centromeric and Telomeric DNA Sequences Differ in Their Attachments

We found that centromeric and telomeric DNA sequences show great differences in their interactions with the nucleoskeleton. The majority of centromeric alpha-satellite repeats are removed during the extraction procedure; i.e., they cannot be anchored by a massive number of attachment sites to the nucleoskeleton (see also Jackson et al., 1996; Craig et al., 1997). The general attachment pattern observed was not connected with the activity of cell metabolism as the results were identical in both unstimulated and stimulated lymphocytes. However, it is possible that a minority of the centromeric sequences is still attached as Strissel et al. (1996) mapped SARs on chromosomes 1 and 16, and were able to hybridize the SAR fraction to the centromeres of the other mitotic chromosomes.

In contrast, telomeric chromatin is tightly attached to the nucleoskeleton in unstimulated as well as stimulated human lymphocytes. This observation is in agreement with

the outcome of studies on nuclear matrix preparations of several established cell lines (de Lange, 1992). In two studies on further characterization of the sequences retained in nuclei after nucleoskeleton preparation, telomeres or telomeric repeats were not described (Jackson et al., 1996; Craig et al., 1997). The reason for this difference to the findings presented here might be found in different methods of evaluation used after the electroelution step.

Our results on nucleoskeleton attachment of alpha-satellite repeats and telomeric repeats prove that two genomic elements can vary dramatically in their attachment properties despite of the fact that they are both transcriptionally silent.

Moreover, it seems that the attachment of telomeric and alpha-satellite repeats is similar throughout interphase. It is known that unstimulated lymphocytes are in G0 phase of the cell cycle (Wachtler et al., 1982). In our samples of stimulated lymphocytes, we counted that ~58% of the cells were in G1 and 40% were in S and G2 phase (~2% were mitotic figures). Nevertheless, the amount of electroeluted alpha-satellite and telomeric sequences was similar in both samples of lymphocytes.

It has been reported that the different chromatin-depletion protocols preferentially retain either structural (nuclear matrix, nuclear scaffold) or functional (nucleoskeleton) sequences (Jackson et al., 1996; Craig et al., 1997). This may explain discrepancies of our findings to reports about nuclear scaffold attachment of centromeres (Strissel et al., 1996). However, in the case of telomeres, our results are comparable to the study of Ludérus et al. (1996), although different preparation techniques were used (nucleoskeleton, nuclear scaffold).

The nature of the attachments of centromeres and telomeres to the nucleoskeleton is not yet fully understood. As both centromeres and telomeres are transcriptionally inactive, the attachment sites cannot be attributed to anchoring via transcriptional complexes. de Lange (1992) suggested that a nucleoprotein complex containing TTAGGG repeats could be the element responsible for nuclear scaffold attachments as TTAGGG repeats introduced by DNA transfection did not behave as matrix-attached loci. The exact nature of these interactions still remains to be defined. However, we can conclude that telomeres rather than centromeres contribute to the formation of intranuclear order by being anchored to the nucleoskeleton.

Activation Is Correlated to Spatial Rearrangement of rDNA Elements

The interaction of ribosomal genes with the nucleoskeleton is of a more complex nature. (a) Clusters of nontranscribed ribosomal genes, which are found outside the nucleolus in unstimulated human lymphocytes (Wachtler et al., 1986), are completely removed during the extraction procedure. (b) When comparing the attachment pattern of the transcription unit (A fragment) and the intergenic, nontranscribed spacer (D_{HH} fragment), considerable differences between unstimulated and activated lymphocytes can be observed. In unstimulated lymphocytes, the amount of D_{HH} fragment removed during extraction is significantly higher than the amount of A fragment. Blot hy-

bridization confirmed the results obtained by in situ analysis. A fraction of cells exists with weaker signal than seen in control cells, or with no signal at all for both the A and D_{HH} fragments. The cell fraction without hybridization signal is larger for the D_{HH} fragments. In activated lymphocytes, the amount of A and D_{HH} fragments eluted is roughly the same.

The results on unstimulated lymphocytes can be partly explained by an attachment via the transcription complexes (Dickinson et al., 1990; Hozák et al., 1994; Weipoltshammer et al., 1996b). Within the extranucleolar ribosomal gene clusters, no transcription takes place (Wachtler et al., 1986). As expected, the transcription unit (A) and the intergenic spacer (D_{HH}) are extracted completely. Within the nucleolus, a preferential extraction of the D_{HH} fragment is observed.

Nevertheless, a certain amount of D_{HH} fragment remains within the nucleolus. Furthermore, in stimulated lymphocytes, the amount of A and D_{HH} fragment removed during the extraction procedure is roughly the same.

These results imply that additional mechanisms of attachment of rDNA to the nucleoskeleton must be of importance. It could, for instance, be assumed that the permanent attachment sites (matrix attachment regions, MARs) that organize the DNA into loops are responsible for at least part of the attachments. MARs have been found preferentially within the intergenic spacers by several authors (Smith and Rothblum, 1987; Stephanova et al., 1993; Gonzales and Sylvester, 1995). One also has to bear in mind that unstimulated lymphocytes are in G0 phase of the cell cycle, whereas stimulated lymphocytes consist of cells in various phases of cell cycle. This could explain the somewhat unexpected finding that the rDNA fragments of stimulated lymphocytes are not significantly more retained in nuclei than those of unstimulated cells. Therefore, in contrast to telomeric and alpha-satellite repeats, a cell-cycle dependence of the rDNA attachment to the nucleoskeleton cannot be ruled out at this point.

In addition, differences might exist in nucleoskeleton attachment of ribosomal genes between different cell types studied. As we have shown in Weipoltshammer et al. (1996b), in nucleoskeleton preparations of HeLa cells, the A fragment remains in the nucleus, whereas the D_{HH} fragment is almost completely removed. All these nucleoskeleton preparations were performed several times with each cell type (unstimulated/stimulated lymphocytes, HeLa cells growing in suspension), and the results were highly reproducible. The reasons for these differences are unknown. However, one can speculate that the differences are due to the cell types studied. In contrast to lymphocytes of the peripheral blood, HeLa cells are malignant, long-term cultured cells. They show alterations in genome (more rDNA gene repeats present and presumably different transcriptional activity of ribosomal genes).

Thus, the nucleoskeleton attachment characteristics of ribosomal genes are dependent on the level of transcriptional activation of ribosomal genes. The results obtained by our study cannot be explained only by one model of nucleoskeleton attachment. Concerning the nature of the interaction of ribosomal genes with the nucleoskeleton, it is most probable that the attachment characteristics we observe result from a combination of sequence-specific DNA/nucleoskeleton attachments (attached either directly or via a mediating protein complex), and of functional attachments, mediated above all by RNA polymerase I transcriptional complexes.

Conclusions

The results demonstrate that: (a) the various repetitive DNA sequences differ significantly in their intranuclear anchoring, (b) telomeric rather than centromeric DNA sequences form stable attachments with the nucleoskeleton, and (c) the activation of nucleolar transcription is connected with a spatial rearrangement of specific rDNA elements relative to the nucleoskeleton. In conclusion, we can observe that very stable DNA/nucleoskeleton attachment sites exist that seem to be independent of cell type and activation state of the cell such as the telomeres (Ludérus et al., 1996). It can be speculated that telomeres play an important role in the formation of chromosome order in interphase nuclei. Vice versa, the majority of the centromeric alpha-satellite sequences can be removed from the nucleus independent of the activation state of the cell. There also exist, however, DNA stretches, like the ribosomal gene repeats, where an attachment to a nucleoskeleton is not only dependent on the activation state of the DNA fragment/gene in question (actively transcribed versus silent genes), but probably also on the cell type. Furthermore, our results on ribosomal genes indicate that more than one attachment mechanism has to be taken into account. Additional studies will be necessary to understand properly the nature of these attachment mechanisms and their functional significance.

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