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SPECIFIC BINDING OF ALU SEQUENCES BY HELA NUCLEAR EXTRACTS

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SUMMARY: Plasmid Blur 8 which contains the 300bp human Alu consensus sequence and plasmid pBR322 were digested with restriction enzymes and the fragments obtained end labelled with ^{32}P-γ-ATP. The end labelled fragments were incubated with HeLa nuclear extracts and the incubation mixtures passed through a nitrocellulose filter. The 300bp alu consensus sequence was preferentially retained on the filter. The HeLa nuclear extract did not preferentially bind any fragments generated from pBR322 and histones which bind nonspecifically all DNA fragments did not preferentially bind the alu sequence. We conclude that the HeLa nuclear extract contains components which specifically bind the human alu sequence.

The Alu family of short, repeated, interspersed DNA sequences is the predominant species of moderately repetitive DNA present in the genomes of primates and rodents (1-3), accounting for a minimum of 3 - 6% of the human genome (2-4). Named for a common Alu restriction site, this group of sequences ranges from 130 base pairs in rat to some 300 base pairs in in humans, and is characterized by such features as genetic mobility, (5,6) and the ability to be transcribed by RNA polymerase III (7-10). Nevertheless, the precise in vivo role(s) of this highly abundant sequence, whether structural or functional, remains unknown. In many instances the structural and functional aspects of the genome can be understood in terms of the interaction of the DNA sequence in question with specific proteins. While the major chromosomal proteins, the histones, do not bind specifically to any type of DNA sequence, the nucleus does contain proteins which bind preferentially to single copy sequences or to DNA with altered conformation. Here we report that the nuclei of human HeLa cells contain a protein component which specifically recognizes Alu sequences.

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MATERIALS AND METHODS

Nuclear proteins were isolated from purified HeLa nuclei essentially as described by Nordheim et al (12). The nuclei were extracted with buffer A (20 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 1 mM dithiothreitol, 0.1 mM PMSF, 0.1% Aprotinin) and sonicated. After bringing the NaCl concentration to 1.5 M, the mixture was centrifuged and the DNA precipitated by making the supernatant 0.4% in polyethyleneimine. After centrifugation the supernatant was made 70% in $(NH_4)_2SO_4$ and centrifuged. The pellet was suspended in buffer B (5 mM Tris, pH 8.0, 0.15 M NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM PMSF, 5% glycerol), mixed with sonicated E. coli DNA which was covalently bound to Sepharose 4B, and the mixture was dialysed. The dialysate was centrifuged and the supernatant, containing protein at a concentration of approximately 30 ug/ml, stored at -20°C.

RESULTS AND DISCUSSION

Our search for Alu binding proteins in the HeLa nuclear extract, employed a nitrocellulose filter binding assay (11-13). Plasmid Blur 8 (a gift from Dr. Carl Schmid) which contains the 300 bp human Alu consensus sequence inserted at the BamHI site of pBR322 (14) was digested with BglI and BamHI to generate five distinct DNA bands (Fig. 1, lane 2). Following

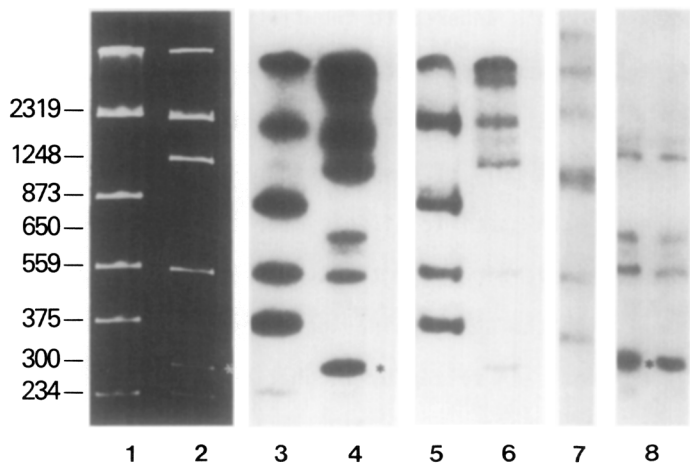

Fig. 1 Specific retention of Alu fragments by HeLa nuclear extracts detected by autoradiographic analysis of ^{32}P-labeled DNA fragments. Lane 1: Ethidium bromide stained polyacrylamide gel revealing the restriction pattern of pBR322 digested with BglI, BamHI and EcoRI. Lane 2: Same, except plasmid Blur 8 was digested with BglI and BamHI. Lane 3: Autoradiograph of a gel containing ^{32}P-end labeled pBR322 fragments. Lane 4: Autoradiograph of the ^{32}P-end labeled fragments derived from Blur 8. Lane 5: Fragments eluted from nitrocellulose filters after pBR322 fragments were incubated with histones. Lane 6: Same as 5 except Blur 8 fragments were used. Lane 7: HeLa nuclear extract was used for binding pBR322 fragments to nitrocellulose. Lane 8: Same as 7 except using Blur 8 fragments. The two experiments shown demonstrate the reproducibility of the observation. Asterisk denotes the 300 bp Alu fragment. Numbers on left denote the size of the fragments in base pairs.

end labeling of both restriction digests with ^{32}P-γ-ATP and polynucleotide kinase, the fragments were used to assay for Alu specific binding components in the nuclear extract prepared from HeLa cells. Various dilutions of the nuclear extracts were incubated with a constant amount of end-labeled fragments derived from the restriction of pBR322 or Blur 8, and the mixture was passed over nitrocellulose filters. Radioactive counting of the quantity of DNA retained on each filter indicated a strong dependence of DNA binding on the presence of HeLa extract. In the absence of nuclear extract about 10% of the input DNA was retained on the filter. By contrast, at a 1:100 dilution of the extract (equivalent to 1.5 ng total proteins) about 20% of the input DNA was bound. We observed a direct correlation between the concentration of nuclear extract and the amount of DNA retained on the filter, up to a 3.5 dilution (0.375 ug proteins) at which point the binding leveled off. At this concentration 72% of the input Blur 8 fragments and 55% of the input pBR322 fragments adhered to the filters. Since the only sequence difference between pBR322 and Blur 8 fragments is the presence of the Alu sequence in Blur 8, our results point to the presence in the HeLa nuclear extract not only of components which bind any type of DNA, but also components with specific affinity for the Alu sequence. The existence of Alu specific binding activity can be demonstrated more conclusively by analyzing the DNA retained on the nitrocellulose filters. The DNA was recovered from each filter by incubation with proteinase K in the presence of 2% SDS for 90 minutes at 37^{o}, followed by ethanol precipitation Lane 8 in Fig. 1 shows the autoradiogram resulting from two different experiments. Comparison of the restriction pattern of DNA applied to the filters (lane 4) to that eluted from the filters (lane 8) clearly indicates that superimposed on some nonspecific binding is a selective enrichment of binding to the Alu fragment. This conclusion is reinforced by the internal control wherein Alu constitutes an intermediate sized Blur 8 fragment. Since the Alu is neither the largest nor the smallest fragment, we have eliminated the possibility that its specific retention on the filter is

due to binding as a function of size. This enhanced binding to Alu gains additional meaning when compared to an external control wherein histones replaced HeLa extract as the protein mediator for DNA filter binding. Histones offer a paradigm of nonspecific binding in that they exhibit a strong affinity for DNA which does not discriminate on the basis of sequence. When a comparison is made between the electrophoretic binding patterns of Blur 8 fragments which were applied to nitrocellulose filters and those which, following incubation with histones, were bound to and eluted from such filters, we observe no enrichment of Alu relative to the other fragments (compare Fig. 1, lanes 4 and 6).

Measurements of the quantity of each fragment applied to and eluted from the filters following interaction with either histones or HeLa nuclear extract were obtained by scanning the autoradiographs of the gels and integrating the area under each peak. The scan reveals close similarities between the relative proportion of each fragment applied to a filter (broken line Fig. 2A) and the relative proportion of the corresponding fragment bound to a filter due to interaction with histones (solid line Fig. 2A). In contrast, the scan of the material eluted from the filters after interaction with HeLa extract (Fig. 2B) shows a marked enrichment in the 300 base pair Alu fragment. A similar experiment performed with end-labeled pBR322 fragments revealed no specific affinity of the HeLa extract for any particular DNA fragment (Fig. 1, lanes 1, 3, 5 and 7). The results obtained from quantitative densitometry of the peaks are summarized in Table 1. In the original restriction digest the Alu fragment constituted 14.9% of the total material, and in the mixture eluted from filters following binding by histones the Alu material comprised 11.0% of of the total. In contrast, Alu accounts for 54% of the material eluted from the filters after binding by HeLa nuclear extract. Furthermore, these are conservative values since the scan did not include the undigested material which failed to penetrate the gel. The ratio of the area under the Alu peak to the area under the adjacent peak (due to the 559 base pair

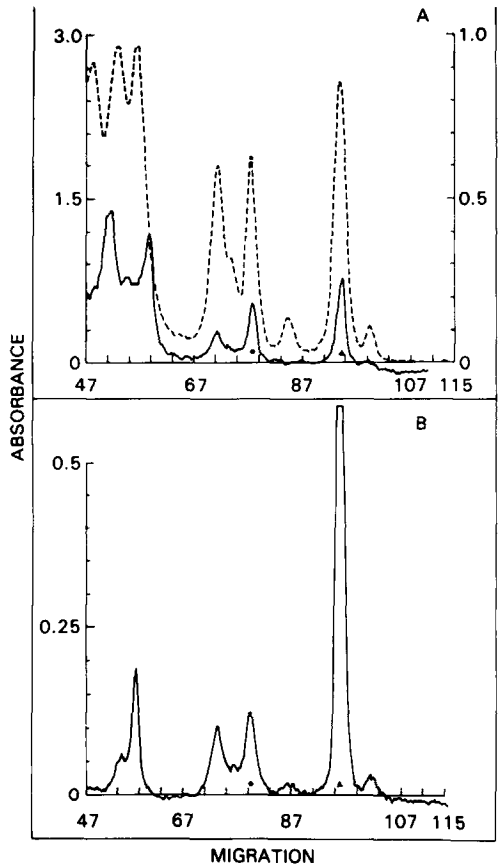

Fig. 2 Densitometric scans of the autoradiograms obtained from gels in which ^{32}P-end labeled restriction fragments derived from Blur 8 were electrophoresed. A) Broken line, original end-labeled fragments (see lane 4, Fig. 1). Solid line, fragments eluted from the filter after binding by histones (Fig. 1, lane 6). B) Fragments eluted from the nitrocellulose filter after binding by HeLa nuclear extract. The Alu fragment is marked with a solid triangle. The adjacent 559 base-pair fragment, with a solid circle.

fragment; marked with a dot in Fig. 2) is 1.55 in the original unbound fragments, 1.41 in the histone bound fragments and 6.47 in the HeLa bound fragments. The binding component(s) in HeLa extract is stable in the presence of RNAse, DNAse and a variety of proteases. However, digestion with pepsin, followed by trypsin, reduced the radioactive counts in plasmid fragments retained by the nitrocellulose filters to 1/3 of those observed for undigested extract. Accordingly, fainter radioactive bands appeared following electrophoresis of eluted Blur 8 and PBR fragments which were originally bound to filters via a predigested HeLa extract than when

Table 1 The 300 bp Alu fragment is preferentially retained on nitrocellulose filters by HeLa nuclear extracts.

		Area (% of Total)		
			Bound by:	
Plasmid	Fragment (bp)	Original	Histones	HeLa Extract
Blur 8	2319	35.3	49.8	5.4
	1248	21.0	23.6	13.3
	650	14.6	6.4	11.9
	559	9.5	8.3	12.9
	300*	14.9	11.0	54.1
	234	1.2	>0.6	>0.6

The autoradiograms of polyacrylamide gels containing separated ^{32}P-end labeled fragments were analysed with a Beckman DU-8 gel scanner. The values given are the % that each peak constitutes of the total area of all peaks. "Original" refers to the total mixture applied to nitrocellulose filters. "Bound by histones" or "HeLa extract" indicates fragments recovered from the filter. For example, the 300 bp Alu fragment constitutes 14.9% of the total fragments. It constitutes 11% of the ^{32}P fragments bound to nitrocellulose after addition of histones and 54% of the fragments bound to the nitrocellulose after addition of HeLa nuclear extract.

the filter binding had been mediated by undigested extract (data not shown). We conclude from these observations that at least a portion of the binding factor(s) in HeLa extract is protein.

Studies on DNA binding proteins have revealed that cells contain an entire spectrum of such protein, ranging from those that are capable of binding to any type of DNA (15), through those which recognize preferentially or exclusively specific DNA conformations (12,13,16,17) or specific DNA segments (13,18-20), including discrete regions localized within genes (21-23). Among the regions shown to be selectively bound by a distinct protein fraction are the clustered, highly repeated sequences comprising the satellite DNA in Drosophila (16,24). We now add to this list a protein component which displays specificity for a highly repetitive but interspersed segment of DNA. Insight into the exact nature of this Alu-binding protein(s) may well come from the knowledge that the 300 base-pair Alu sequence can be transcribed by RNA polymerase III in conjunction with one or more initiation factors (25-29). However the polymerase III

5S gene transcription factor by itself does not cause retention of DNA in the range of 5S on nitrocellulose filters (30) and plymerase III by itself does not bind to DNA. The possible identity of the protein(s) in question with a component of the polymerase III complex or with some protein of totally independent function remains to be elucidated.

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