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ISOLATION AND VISUALISATION OF ALKALI STABLE PROTEIN/DNA COMPLEXES

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Distinct polypeptides, 54,000-68,000 daltons in size, are alkali-stably bound to eukaryotic DNA. DNA fragments several hundred base pairs in length associated with these polypeptides are preferentially retained on glass fibre filters from solutions containing 1 M sodium chloride. About 50 percent of the protein/DNA complexes present in total DNA are retained on filters together with about 2 percent of the DNA. This preferential binding is demonstrated (a) by the ratio of ^{3}H and ^{35}S radioactivity retained on filters after filtration of DNA from [^{3}H]thymidine and L-[^{35}S]methionine labelled cells, (b) radioiodination of the material retained on filters and passing filters respectively and (c) by electron microscopical visualisation of the polypeptide component in the complexes after chemical modification with dinitrofluorobenzene (DNFB) followed by incubation with dinitrophenyl (DNP) specific antibodies.

Introduction

DNA isolated from eukaryotic cells by means of proteases, SDS and phenol [1] still contains peptide materials [2,3]. Most of this residual peptide material can be removed by additional purification steps e.g. phenolisation under vigorous shaking and/or treatment with alkali followed by sedimentation [2-4]. However, distinct polypeptides 54,000 and 68,000 daltons in size are not released from DNA by these techniques [2-4]. This peptide material can only be isolated after degradation of DNA [2,4]. In this paper it will be described how DNA fragments associated with this most tightly bound peptide material can be enriched. The protein material tightly attached to DNA cannot be visualized by electron microscopy directly because the frequency in total DNA is low and the size of this material is too small to be resolved from the background [5]. However, the protein molecules in enriched protein/DNA complex fractions can be indirectly visualized after chemical modification of the polypeptides involved followed by decoration with specific antibodies.

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Methods

Labelling of DNA and isolation: Ehrlich ascites tumor cells were labelled starting at day five after inoculation by three i.p. injections each containing 400 µCi of L-[^{35}S]methionine (1100 Ci/mmole) and 20 µCi of [^{3}H]thymidine (20 Ci/mmole). The intervals between two injections were approximately twelfe hours. A second DNA preparation was [^{3}H]thymidine labelled. In this case tumor bearing animals received one i.p. injection of 100 µCi of [^{3}H]thymidine. Twenty four hours after the (last) injection tumor cells were harvested and DNA was isolated according to the procedure described by Gross-Bellard et al. [1]. The two DNA preparations in use showed A_{260}/A_{280} ratios of 1.80. The specific radioactivity of the L-[^{35}S]methionine, [^{3}H]thymidine-labelled DNA is given in Table 1. The specific radioactivity of the [^{3}H]thymidine-labelled DNA was 21.3 x 10^3 cpm per A_{260} unit.

Filter binding of protein-associated DNA: Sheared DNA (ultrasonic power, 5 x 30 sec) or DNA restriction fragments dissolved in filtration buffer (5 mM Tris, 2 mM EDTA, 1 M NaCl, pH 8.0) were filtered by gravity through Whatman GF/C filters (2.3 cm) supported by a glass filter holder (Millipore). The amount of DNA filtered through one filter was between 0.1 and 0.5 A_{260} units. The filters were soaked in filtration buffer for 30 min prior to filtrations. After the filtration of DNA containing solutions the filters were washed with 1 ml of filtration buffer per 0.1 A_{260} unit of DNA submitted to filtration. Unspecific binding of DNA fragments was determined by refiltration of the first filtrate. The washed filters were dried and counted in scintillation fluid on toluene basis in a Nuclear Chicago scintillation spectrometer (Mark III) with the appropriate program for single [^{3}H] and double label [^{3}H], [^{35}S] counting. The retained material was recovered for radio-iodination and DNFB-reaction from several filters loaded as described above by elution with the following buffer: 1 % SDS, 2 mM EDTA, 10 mM NaCl, 5 mM Tris, pH 8.0 (37°C, shaker). After dialysis against the same buffer (without SDS) the complexes were precipitated with 3 vol of ethanol (4°C). DNA contained in aliquots of similarly dialysed filtrates containing an equal amount of ^{3}H label were also precipitated with ethanol.

Visualisation of protein/DNA complexes was carried out according to a method described in the literature [6]. Briefly, DNA fragments (∿ 10 µg) were incubated in PBS (300 µl) containing 1 mM EDTA, 10 % DMSO and 1 µl of DNFB for two hours at 37°C. Unreacted DNFB was removed by ether extraction and dialysis against PBS/EDTA. The retentate was incubated with 60 µl of anti-DNP/BSA serum (Miles) for two hours at 37°C followed by passing the mixture through a Sepharose CL 2B column (1 ml) equilibrated with PBS/EDTA. Fractions containing the DNA fragments were prepared for electron microscopy by mica-method [7]. Briefly, 12.5-37.5 µl of DNA-solution were mixed with Tris-Mg acetate pH 8.8 to a final concentration of 10 mM each and with glutardialdehyde to a final concentration of 0.1 % to give 125 µl. From this mixture 62.5 µl-droplets were put into plastic petri dishes; after 20 min 1 x 1.5 cm pieces of freshly cleaved mica (Ladd, Burlington, USA) were pressed onto the droplets. The mica sheet with the adherent material was washed in 0.05 % glutardialdehyde in water for 90 min, and briefly in pure water. DNA was stained with uranylacetate (0.2 % in 90 % ethanol), washed with 90 % ethanol and subsequently shadowcast with PT-C under 7°. Electron microscopical examination of specimens was performed in a Philips EM400 (60 kV). The magnification was calibrated by a cross-grating-replica. Length measurements were taken from prints by a device described by Schroeter et al. [5]. In experiments with standard DNA (pBR322) the accuracy of this method proved to be within ± 6 % of the absolute value.

Other methods: Restriction fragments were produced according to the suggestions of the producer of AluI restriction endonuclease (Boehringer). Radio-iodination and SDS polyacrylamide gel electrophoresis were made as described previously [2,3,8].

Table 1

Binding of DNA specific label [^{3}H] and peptide specific label [^{35}S] after filtration of DNA fragments (shear size) through glass fibre filters (1 M sodium chloride). DNA was isolated from cells labelled with [^{3}H]thymidine and L-[^{35}S]methionine. Mean values and standard deviations of six identical samples.

DNA	[^{3}H]cpm x $10^{-3}/A_{260}$	[^{35}S]cpm x $10^{-3}/A_{260}$	$\frac{[^{35}S]}{[^3H]}$
Before Filtration	224.3 ± 3.9	52.0 ± 7.1	0.23
Retained on Filters	5.1 ± 0.1 (≙ 2.27%)	24.9 ± 3.8 (≙ 47.88 %)	4.88

Results

DNA isolated from Ehrlich ascites tumor cells labelled with [^{3}H]thymidine and L-[^{35}S]methionine contains both, ^{3}H- and ^{35}-label (Table 1). After shear degradation of ^{3}H, ^{35}S-labelled DNA and filtration of the fragments in 1 M sodium chloride solution through glass fibre filters the $^{35}S/^3H$-ratio of the material retained on the filters was significantly different from that before filtration. By one filtration 2.27 % of the sheared DNA (^{3}H-label) but 47.88 % of the ^{35}S-label was stably adsorbed at the filters. The amount of the ^{35}S-label and the ^{3}H-label retained on the filters was proportional to the amount of DNA submitted to filtrations up to 0.5 A_{260} units per filter (Fig. 1A). In further filtration steps only a small additional amount of ^{35}S-labelled material which passed the filter in the first filtration step was adsorbed (Fig. 1B). This indicates that the ^{35}S-labelled material passing the filter in the first filtration is not retained in further filtrations. The desorbed and ethanol precipitated material was further characterized by labelling with ^{125}iodine and SDS-polyacrylamide analysis. An equal amount of DNA fragments (on the basis of DNA specific label) from the filtrate was also treated with ^{125}iodine and processed under identical conditions. The densitometer tracings of the films exposed for short times to the gels reveal the characteristic 54,000-68,000 dalton material in gels loaded with the radio-iodinated filter extracts (Fig. 2). In contrast, gels loaded with radio-iodinated material from the

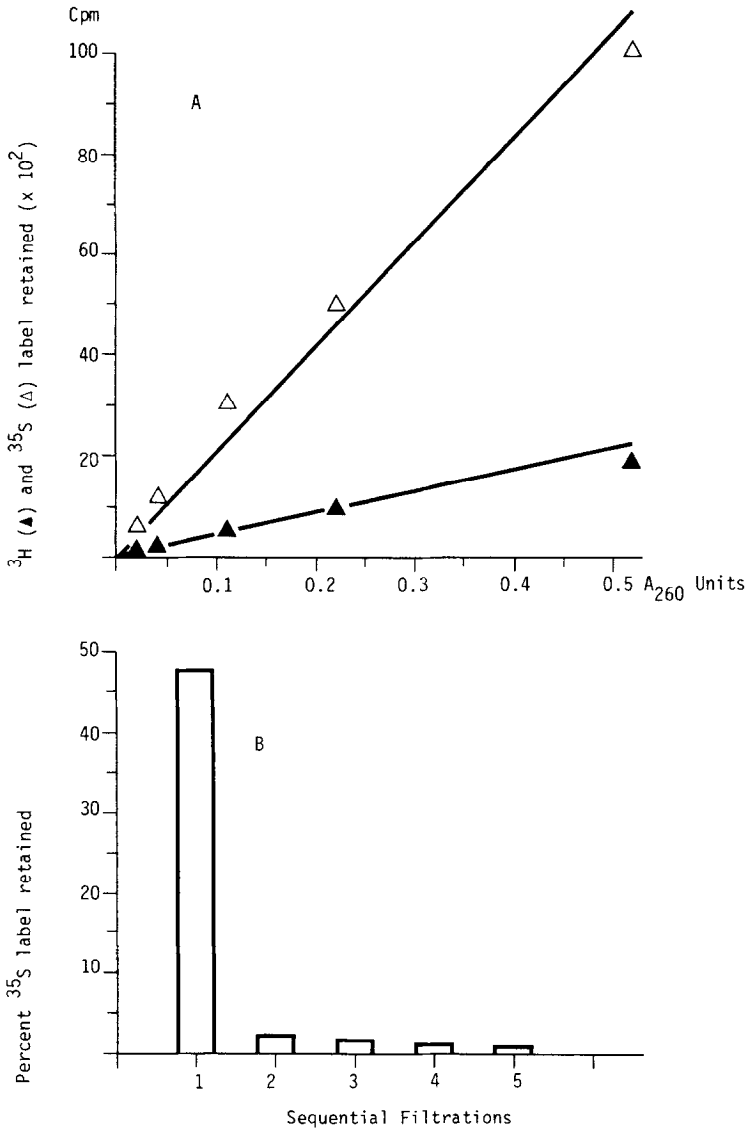

Figure 1

(A) ^3H, ^{35}S label retained on glass fibre filters during filtration of sheared DNA labelled with [^3H]thymidine and L-[^{35}S]methionine in dependence on the amount of DNA submitted to filtration.
(B) ^{35}S label retained on glass fibre filters during repeated filtrations of sheared [^3H]thymidine and L-[^{35}S]methionine labelled DNA.

filtrate had to be exposed to the films for about twenty five times longer until this material could be clearly detected in this fraction. This shows that the concentration of the protein/DNA complexes per DNA in the filtrate is significantly lower. However, since 97.73 percent of the DNA appears in the filtrate this is supporting the result demonstrated in Table 1 indicating that

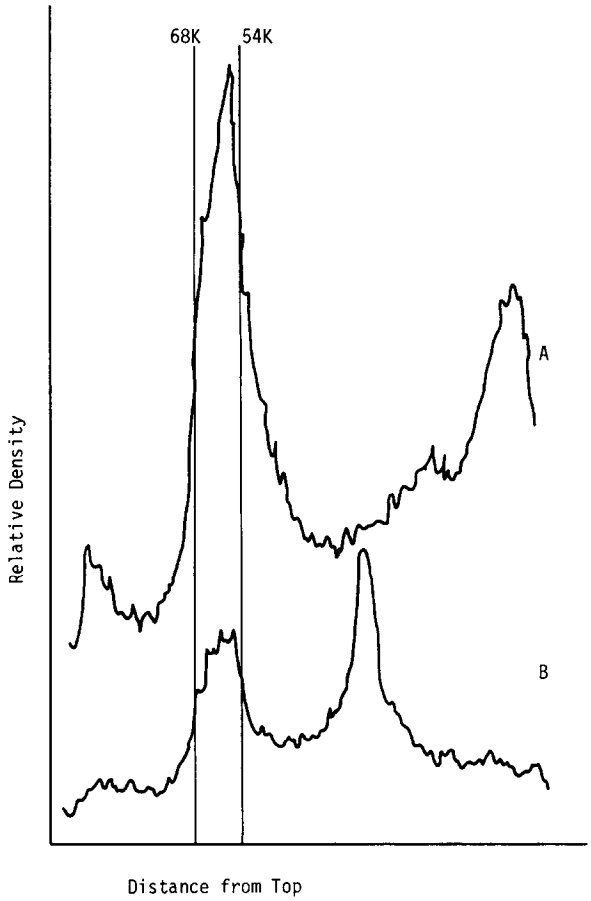

Figure 2

Densitometer tracings of films exposed to SDS-polyacrylamide slab gels after electrophoresis of ^{125}I treated filter extracts (A) and filtrates (B). A solution (1 M NaCl) containing sheared DNA was passed through glass fibre filters. Aliquots of the dialysed filter extract and the filtrate containing equal amounts of DNA (0.36 A_{260} units) were precipitated with ethanol and treated with ^{125}I. The radiolabelled material was analyzed by SDS-polyacrylamide gel electrophoresis. (A) was exposed for 5 min and (B) was exposed for 120 min to the film.

in the first filtration step 50 percent of the peptide material is retained with (2.27 percent) DNA and that 50 percent of the peptide material passes the filter together with (97.73 percent) DNA. Fig. 3 shows that additionally phenolized AluI fragments associated with the 54,000-68,000 dalton material are also preferentially retained on glass fibre filters. This could also be demonstrated by electron microscopical techniques. AluI fragments retained on filters in 1 M sodium chloride solution and fragments passing the filter were reacted with DNFB under conditions under which only peptide meterials become

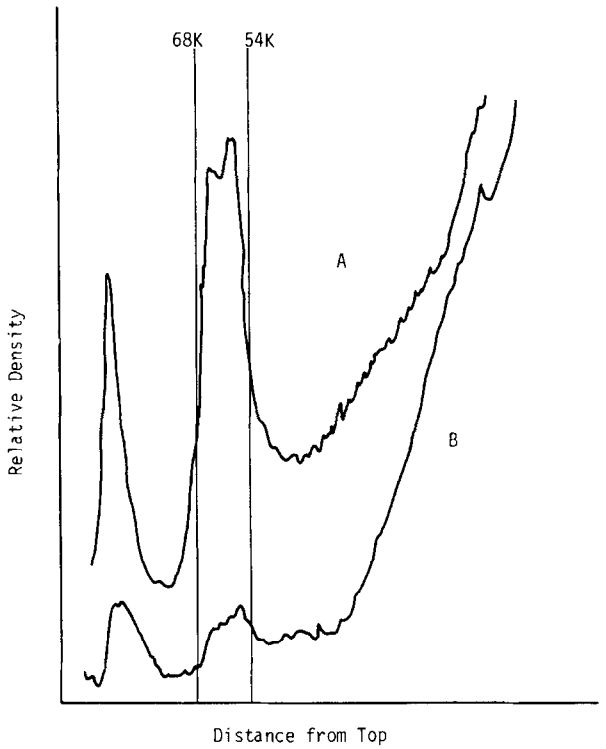

Figure 3

Densitometer tracings of films exposed to SDS-polyacrylamide slab gels after electrophoresis of ^{125}I treated filter extracts (A) and filtrates (B). A solution (1 M NaCl) containing AluI digested DNA was passed through glass fibre filters. Aliquots of the filter extracts and of the filtrates containing equal amounts of DNA (0.044 A_{260} units) were radiolabelled with ^{125}I and analyzed as described under Fig. 2. (A) and (B) were exposed to the film for 60 min.

DNP-substituted. Specific DNP-antibodies were then used to detect DNP-substituted sites. Figs. 4A and B show the molecular morphology of AluI fragments submitted to this procedure. There is no significant difference in the length distribution of filter bound and unbound molecules (Table 2). However, DNA molecules associated with the DNP-specific antibodies can be easily distinguished from those which are not associated with antibodies. The fragments desorbed from the filters frequently contained one of the typical protein knobs whereas in fragments passing the filter the protein knobs were visible only occasionally (Table 3). However, since the filtrate contains about 43 times more DNA the total amount of markers in the material retained on filters and in that passing the filter is about equal supporting again the conclusion that

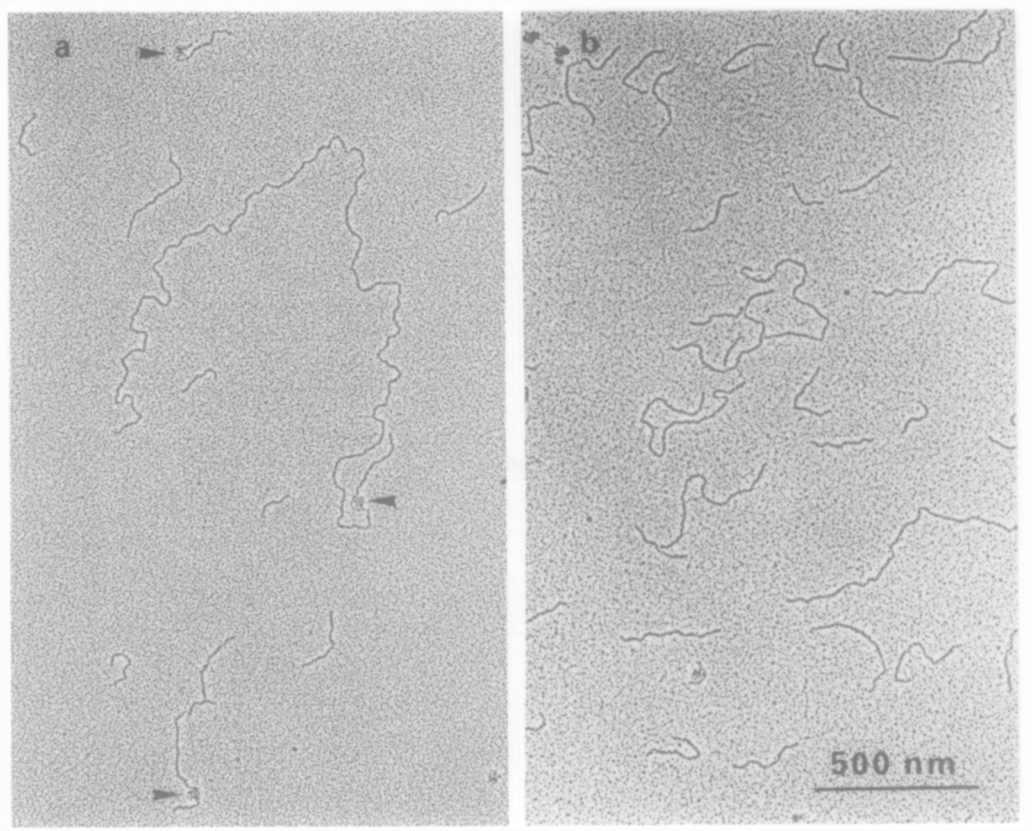

Figure 4

AluI fragments of DNA bound to glass fibre filters in presence of 1 M sodium chloride (A) and fragments passing the filter under these conditions (B) after reaction with DNFB and DNP specific antibodies. Antibody markers in (A) are indicated by arrowheads; in (B) no markers are visible. Magnification: 50,000 x.

Table 2

Length distribution of AluI fragments of Ehrlich ascites tumor cell DNA established by electron microscopy.

	Number of molecules traced	Average length (µm)	Distribution (µm)	Most frequent size (50 percent of the molecules (µm)
Filtrate	759	0.2811	0.02-2.94	0.2-0.3
Filter	462	0.3401	0.03-4.91	0.2-0.3
Total	1221	0.3034*	0.02-4.91	0.2-0.3

*From this value the average molecular weight of one AluI fragment can be calculated: 0.6068×10^6 d.

Table 3

Frequency of antibody associated DNA molecules after reaction of filter bound and unbound material with DNFB and DNP specific antibodies.

	Traced DNA (μm)	Number of markers	Average length of DNA (μm) per marker
Filter bound material	217	41	5.3
Material passing the filters	282	2	141

50 percent of the complexes are retained together with a small amount of DNA on the filters. This was used for a rough estimate of the frequency of protein/DNA complexes in DNA (Table 4).

Discussion

In this paper we show that DNA fragments several hundred base-pairs in size which are associated with the most tightly bound peptide material can be enriched by a filter binding technique which was originally developed for the analysis of adenovirus DNA terminal protein complex [9]. Other protein/DNA complexes such as topoisomerase/DNA complexes have also been successfully isolated by this technique [10]. Thus, it appears that this filter binding assay

Table 4

Estimation of the frequency of protein/DNA complexes under the assumption that 50 percent of protein associated AluI fragments are retained on filters. Mean values and standard deviations of nine identical samples submitted to filtration; each containing 3150 cpm DNA specific label.

DNA Specific Label	[cpm] ± S.D.
Retained (first filtration):	128.8 ± 24.8
Retained (second filtration):	21.0 ± 6.7
Specific radioactivity due to 50 percent absorption of protein/DNA complexes:	107.8
Radioactivity to be expected if 100 percent of protein/DNA complexes were retained:	215.6
Number of AluI fragments between two complexes:	3150/215.6 = 14.61
Average molecular weight* of DNA between two complexes:	$14.61 \times 0{,}6068 \times 10^6$ d = 8.81×10^6 d

*See footnote of Table 2.

is generally applicable for the isolation of protein/DNA complexes which are stable under the filtration conditions. However, in contrast to DNA fragments associated with adenovirus terminal protein [9] or topoisomerases [10] the complexes between the 54,000-68,000 dalton polypeptide and DNA are not absolutely quantitatively but highly preferentially retained on glass fibre filters. This suggests that the interaction of these complexes with the binding sites of glass fibres is somewhat weaker than the interaction of the complexes investigated earlier by this method. Thus, although it is possible to accumulate these complexes by filtrations through glass fibre filters the significance of the frequencies estimated by this assay has to be discussed. The exact percentage of the protein associated fragments retained on the filters is unknown. However, our results show that approximately fifty percent of the fragments associated with protein are retained by one filtration. Under this assumption the average frequency is one per 8.87×10^6 daltons. This frequency corresponds well with protease-inducible alkali-labile sites in eukaryotic DNA estimated by centrifugation (8.5×10^6 daltons, [11-13]) and electron microscopy (6.9×10^6 daltons [5]).

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