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Proteins Tightly Bound to HeLa Cell DNA at Nuclear Matrix Attachment Sites

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DNA-protein complexes have been isolated from HeLa cell nuclei and nuclear matrix preparations. Two proteins, 55 and 66 kilodaltons in size, remain bound to HeLa DNA after treatment at 80°C in 2% sodium dodecyl sulfate and purification by exclusion chromatography on Sepharose 2B-CL in the presence of 0.3% sodium dodecyl sulfate. These proteins appear to be tightly bound but not covalently linked to the DNA, and they are distributed over the DNA with an average spacing of 40 kilobase pairs. This spacing distribution remains essentially constant throughout the cell cycle. The proteins are bound to the residual 2% of HeLa cell DNA which remains attached to the nuclear matrix after extensive nuclease digestion, a condition which reduces the average size of the DNA to ~150 base pairs. Our results suggest that these tightly bound proteins are involved in anchoring cellular DNA to the nuclear matrix. These tightly bound proteins are identical by partial peptide mapping to proteins found tightly bound to the DNA of mammalian, plant, and bacterial cells (D. Werner and C. Petzelt, J. Mol. Biol. **150**:297–302, 1981), implying that these proteins are involved in the organization of chromosomal domains and are highly conserved in both procaryotic and eucaryotic cells.

The DNA of eucaryotic cells is organized into structural domains 30 to 90 kilobases (kb) in size. These domains are seen in metaphase chromosomes as loops or rosettes of DNA bound to a protein scaffold (10, 40, 43). In interphase nuclei the DNA is organized in domains of supercoiling measured at 84 to 94 kb (4, 22, 57), and the DNA is attached to the nuclear matrix with an average spacing of between 35 and 60 kb (25, 48). Cellular DNA replication also takes place in association with the nuclear matrix (16, 34, 42, 58), and eucaryotic DNA replicons have a size distribution similar to that of the structural domains described above (18). Several proteins that are tightly bound to cellular DNA have been implicated in maintaining supercoiled domains, binding DNA to chromosome and nuclear scaffolds, or binding to transcriptionally active genes (3, 24, 28, 37, 46, 47; S. Matsui, G. Antoniades, J. Bagler, R. Berezney, and A. A. Sandberg, J. Cell Biol. **91**:60a, 1981). However, proteins that mediate the interaction between cellular DNA and the nuclear matrix have not been defined.

Using methods initially developed to study the adenovirus terminal protein-DNA complex (14, 21, 50), we isolated a stable DNA-protein complex from HeLa cells that contains two proteins, 55 and 66 kilodaltons (kd) in size. These proteins are tightly bound to cellular DNA and exhibit a constant average spacing of about 40 kb throughout the cell cycle. They are specifically enriched in the nuclear matrix fraction, even after 98% of the cellular DNA is removed by nuclease digestion and the size of the bulk DNA is reduced to approximately 150 base pairs (bp). These proteins also possess peptide map homology to proteins previously reported to be bound to a wide variety of procaryotic, eucaryotic, and plant cells DNAs, even after alkali treatment, pronase digestion, and phenol extraction (7, 28, 60–63). These observations strongly indicate that the HeLa cell proteins are involved in anchoring DNA on the nuclear matrix and that similar proteins participate in the organization of chromosomal domains in both procaryotic and eucaryotic cells.

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MATERIALS AND METHODS

Cells. HeLa S_3 cells were grown in suspension culture at a concentration of 3×10^5 cells per ml with F-13 medium (GIBCO Laboratories) supplemented with 7% fetal calf serum. Cells were labeled for 24 h with $^{32}P_i$ (carrier-free; New England Nuclear Corp.) at a concentration of 1 μCi/ml in phosphate-free F-13 medium containing 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) and 2% fetal calf serum.

HeLa S_3 cells were grown as monolayers with Dulbecco minimal essential medium supplemented with 10% fetal calf serum. Cells were labeled for 65 to 70 h with $^{32}P_i$ at a concentration of 40 μCi/ml in the same medium. Alternatively, cells were labeled for 5 h with [^{3}H]thymidine at a concentration of 10 μCi/ml in Dulbecco minimal essential medium after 30 min of thymidine depletion with 10^{-3} M fluorodeoxyuridine.

Buffers. TE buffer is 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5). NB buffer is 10 mM Tris-hydrochloride (pH 7.4)–1 mM $CaCl_2$–250 mM sucrose–0.1 mM phenylmethylsulfonyl fluoride. PBSd buffer is 170 mM NaCl–30 mM KCl–5 mM $Na_2HPO_4 \cdot 7H_2O$–18 mM KH_2PO_4 (pH 7.2). Scaffold buffer A is 100 mM NaCl–50 mM KCl–20 mM Tris-hydrochloride (pH 7.5)–0.1 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride–10% glycerol (vol/vol). Scaffold buffer B is 100 mM NaCl–20 mM Tris-hydrochloride (pH 9.0)–20 mM EDTA–0.2% Nonidet P-40 (NP-40) (Shell Chemical Corp.)–10% glycerol. Scaffold gradient buffer is 10 mM Tris-hydrochloride–(pH 9.0) 2 M NaCl–10 mM EDTA–0.1% NP-40.

Isolation of nuclei. Cells were washed with NB buffer and disrupted with 0.5% NP-40 in NB buffer. The lysate was centrifuged at $1,000 \times g$ for 10 min; the nuclear pellet was washed with NB buffer containing 0.5% NP-40, suspended in NB buffer at a concentration of 3×10^7 nuclei per ml, and digested with pancreatic RNase and T_1 RNase (both at 20 U/ml) for 30 min at 37°C.

Alternatively, nuclei were prepared as described by Robinson et al. (50). Washed cells were suspended at 10^7 cells per ml in buffer containing 140 mM NaCl, 20 mM Tris-hydrochloride (pH 7.5), 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.05% Triton X-100. After 10 min on ice, cells were broken with six strokes in a Dounce homogenizer equipped with a tight-fitting B pestle. The lysate was then centrifuged at $1,000 \times g$ for 10 min. The nuclear pellet was suspended in Scaffold buffer A and digested with RNase as above.

Cell synchronization. Cells were synchronized by a double thymidine block as described by Thilly (56). HeLa S_3 cells grown as monolayers were seeded about 20% confluent and grown in Dulbecco minimal essential medium plus 10% fetal calf serum with the following medium changes: (i) 12 h with 0.2 mM thymidine, (ii) 12 h thymidine free, (iii) 12 h with 0.2 mM thymidine, (iv) 12 h thymidine free plus $^{32}P_i$ (2 μCi/ml), (v) 12 h with 0.2 mM thymidine, and (vi) thymidine free until harvested.

Preparation of nuclear matrix. Nuclear matrix was prepared by the method of Razin et al. (48). HeLa cells were grown as monolayers in $^{32}P_i$ (20 μCi/ml) for 65 to 70 h. Cells were washed with PBSd buffer, suspended at a concentration of 3×10^6 cells per ml in Scaffold buffer A, and lysed by the addition of 0.5% NP-40 and 0.1% Triton X-100. After 15 min on ice, the nuclei were washed twice with Scaffold buffer A and finally suspended in Scaffold buffer A at a concentration of 10^7 nuclei per ml. Nuclei were digested with restriction endonucleases as described below. For micrococcal nuclease digestion, nuclei were adjusted to 10 mM $CaCl_2$ and incubated with the enzyme (Worthington Biochemicals Corp.; 1 U/ml) for 10 min at 37°C. Reactions were stopped by diluting solutions fivefold into Scaffold buffer B on ice. Samples were adjusted to 2 M NaCl and incubated on ice for 45 min to extract soluble DNA not attached to the matrix. Samples were layered on step gradients constructed with a 5-ml cushion of 50% sucrose (wt/vol) in Scaffold gradient buffer under 25 ml of 15% glycerol (vol/vol) in Scaffold gradient buffer. The gradients were centrifuged in Spinco SW28 tubes at 15,000 rpm for 45 min at 2°C. As described by Razin et al. (48), the extracted nuclei (nuclear matrix) collected on the top of the 50% sucrose cushion, and the soluble DNA remained at the top of the gradient. Fractions containing nuclear matrix and soluble DNA were pooled separately, and each pool was incubated with RNase (25 μg/ml) for 1 h at 37°C. Nuclear matrix and soluble DNA were separately pelleted by centrifugation at 45,000 rpm for 4 h at 20°C in a Spinco SW50.1 rotor. Pellets were dissolved in sodium dodecyl sulfate (SDS) buffer as described above.

Column chromatography. Chromatography on Sepharose 2B-CL or 4B-CL (Pharmacia Fine Chemicals, Inc.) was done as previously described (21, 50). DNA-protein mixtures were heated at 80°C for 2 to 3 min and loaded on columns in buffer containing 10 mM Tris-hydrochloride (pH 7.9), 150 mM LiCl, 10% glycerol, 2% SDS, 50 mM dithiothreitol, and 0.001% bromphenol blue. The columns were then eluted either with a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, 1 mM mercaptoethanol, and 0.05% SDS or with a buffer containing 10 mM Tris-hydrochloride (pH 7.9), 150 mM LiCl, 0.5 mM EDTA, and 0.3% SDS.

Filter-binding assay. The filter-binding assay used to analyze protein-DNA complexes was done as previously described (14, 15).

Enzymes. The purification and assay conditions of restriction endonucleases *Eco*RI, *Bam*HI, *Sma*I, and *Hae*III have been described (35). Pancreatic RNase A (Sigma Chemical Corp.) was heated in TE buffer to 100°C for 5 min at a concentration of 5 mg/ml before use. Pronase (grade B; Calbiochem) was incubated in TE at 37°C for 2 h at a concentration of 10 mg/ml before use.

Radioactivity determinations. After samples were adjusted to 5% trichloroacetic acid on ice, acid-insoluble precipitates were collected on GF/C glass-fiber filters. The dried filters were counted by liquid scintillation spectrometry with a toluene-PPO(2,5-diphenyloxazole)-POPOP[1,4-bis-5-phenyloxazolyl)benzene] mixture (44). When ^{32}P was the sole isotope, radioactivity was determined by Cerenkov counting.

Gel electrophoresis. Agarose gels were run as previously described (35). Polyacrylamide gels (10 and 15%) were run on a microslab gel apparatus (32) with the modifications of Tatchell and Van Holde (55) and the buffer system of Laemmli (29). Gels were stained with Coomassie blue or silver stained by the method of

Morrissey (36). Partial peptide maps were done by the method of Cleveland et al. (8).

RESULTS

Purification of a stable HeLa cell DNA-protein complex. Randomly growing HeLa cells were labeled with $^{32}P_i$ for 24 h, and nuclei were prepared as described above. After a digestion with RNase the purified nuclei were lysed with 0.5% SDS and heated to 80°C for 2 min to hydrolyze [^{32}P]RNA. After mild shearing to reduce the viscosity of the lysate, HeLa DNA and potential DNA-protein complexes were separated from nonassociated proteins by chromatography on a Sepharose 2B-CL column in the presence of 0.05% SDS as described by Robinson et al. (50). The DNA in the void-volume fractions, which represented most of the total cell DNA, was pooled, and the SDS was removed by dialysis against TE buffer. The DNA sample was then assayed for the presence of tightly bound proteins (TBPs) with both a filter-binding assay and a gel exclusion assay.

Previous studies on the adenovirus DNA-terminal protein complex had shown that this DNA-protein complex was quantitatively retained on glass-fiber filters in the presence of high concentrations of salt (14, 15). Up to 80% of the ^{32}P-labeled HeLa cell DNA in the void-volume fractions of the Sepharose 2B-CL column was also retained on glass-fiber filters; the percentage of the DNA retained was directly proportional to the fragment size of the DNA before chromatography. Binding of the HeLa cell DNA-protein complex was also salt dependent: although less than 3% of the counts were bound in TE buffer, the amount of DNA bound increased with increasing amounts of salt and reached a maximum as the salt concentration was raised above 0.5 M NaCl (Fig. 1). Protein-free adenovirus [^{3}H]DNA that was mixed with the ^{32}P-labeled HeLa DNA-protein complex exhibited little binding (<0.5%) at all salt concentrations used. Retention of the ^{32}P label on glass-fiber filters was not diminished by further RNase treatment, but it was completely abolished by treatment with DNase I. Binding was also markedly reduced by prior digestion with proteases: pronase, chymotrypsin, and trypsin reduced binding to an average of 4.4%, 2.3%, and 6.2%, respectively. The residual binding of protease-treated complexes most likely reflects the high resistance of the TBPs to protease digestion (see below).

The HeLa DNA-protein complex, like the adenovirus DNA-terminal protein complex (6, 53), did not enter agarose gels during electrophoresis. A significant fraction of the ^{32}P radioactivity remained at the origin when ^{32}P-labeled HeLa DNA-protein complex was electrophoresed on a 1% agarose gel before proteolysis (Fig. 2, lane 1). Treatment of the complex with chymotrypsin (lane 2) or trypsin (lane 3) markedly reduced the amount of the ^{32}P-labeled DNA remaining at the top of the gel. The filter-binding and gel exclusion assays thus gave similar results, both qualitatively and quantitatively, which indicated that HeLa cell DNA did indeed have proteins bound tightly to it.

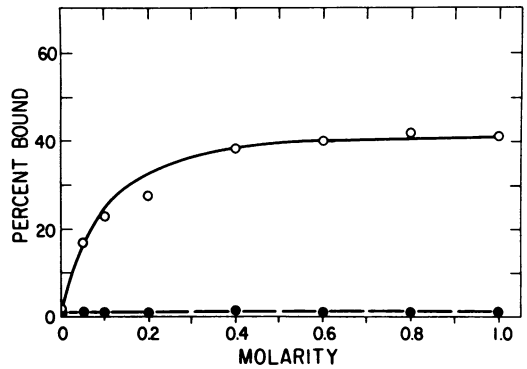

FIG. 1. Effect of NaCl concentration on the binding of the HeLa DNA-protein complex to glass-fiber filters. Samples of ^{32}P-labeled HeLa complex (○) were mixed with ^{3}H-labeled adenovirus DNA (●), adjusted to the indicated concentration of NaCl, and passed through glass-fiber filters prewashed with the same concentration of NaCl in TE buffer.

Arrangement of TBPs along HeLa DNA. The TBPs associated with HeLa DNA could either be organized in a close-packed array or have a long-range spacing. We distinguished between these two extreme models of arrangement by analyzing the fraction of DNA bound to protein as a function of DNA size. If the proteins were clustered on a small subset of the DNA fragments, the fraction of DNA bound in the filter-binding assay would not be changed significantly as the bulk DNA pool was cleaved into smaller fragments. However, the long-range spacing model predicts that as the size of the DNA is decreased, fewer and fewer DNA segments will bind the filter as fewer segments will have protein associated with them. The data in Table 1 clearly support the second model and indicate that TBPs are spaced along the DNA with an average interval of 40 ± 11 kb. In these experiments, DNA was cleaved with different restriction endonucleases to vary the molecular weight of the cellular DNA, and the average molecular weight of DNA in each preparation was determined by analytical velocity sedimentation. The spacing interval was calculated from Poisson statistics, assuming that one or more TBPs was sufficient to bind any DNA fragment to the filter. The estimates for the spacing interval were remarkably similar, considering that the fraction

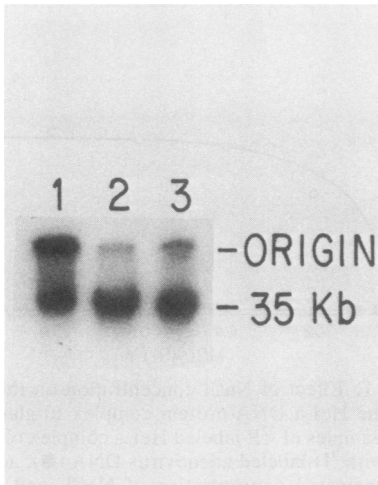

FIG. 2. Agarose gel electrophoresis of the HeLa DNA-protein complex. ^{32}P-labeled HeLa DNA-protein complex was electrophoresed on a 1% agarose slab gel, dried, and autoradiographed. Lane 1, Undigested HeLa DNA-protein complex; lane 2, HeLa DNA-protein complex digested with chymotrypsin (0.5 mg/ml) for 1 h at 37°C; lane 3, HeLa DNA-protein complex digested with trypsin (0.5 mg/ml) for 1 h at 37°C. The average size of the HeLa DNA used in this experiment was 35 kb.

of the total DNA that bound to the filter varied by 30-fold and that the average molecular weight of the DNA varied by 60-fold.

We also determined the spacing of the proteins as a function of the cell cycle. Cells were synchronized by using 0.2 mM thymidine (56) and labeled with $^{32}P_i$. The cells were harvested after release of the double thymidine block, and the nuclei were isolated (50), digested in situ with *Eco*RI (or *Hae*III) and RNase, disrupted in 2% SDS at 80°C for 3 min, and chromatographed on Sepharose 4B-CL (21). After dialysis to remove the SDS, each sample was electrophoresed on a 1% agarose gel and exposed for autoradiography. The percent of DNA retained at the origin, the average molecular weight of the DNA, and the average protein spacing were determined from a densitometric analysis of the autoradiogram. The results (Fig. 3) again demonstrated that the TBPs were distributed at periodic intervals along the DNA and that the average spacing (48 ± 8 kb) remained relatively constant throughout the cell cycle. However, because of the relative imprecision of this analytical method, we cannot be certain that some subtle changes in the spacing distribution occurred during the period of maximum mitotic activity (10 to 14 h after the cells were released from the thymidine block).

It was possible that the TBPs could have dissociated during the 80°C heat treatment before chromatography and then reassociated randomly on the DNA when the temperature was reduced. Since this possibility could have invalidated the spacing data discussed above, a mixing experiment was done to directly monitor the exchangeability of the TBPs onto an exogenously added DNA. HeLa cells were labeled with $^{32}P_i$ and nuclei were isolated as before. At this point, deproteinized ^3H-labeled HeLa DNA was added to the nuclear fraction. The mixture was heated at 80°C for 3 min in the column sample buffer (containing 2% SDS), and HeLa DNA-protein complexes were isolated by exclusion chromatography. The filter-binding assay was then used to detect the presence of proteins that exchanged onto the added [^3H]DNA. In this experiment, the quantities of [^3H]DNA and [^{32}P]DNA were about equal, and the average size of the [^3H]DNA was 10 times that of the [^{32}P]DNA. If appreciable protein exchange had occurred, we would have expected a significant increase in the amount of [^3H]DNA retained on the glass-fiber filter. However, the retention of the [^3H]DNA on the filter was not increased, nor was the retention of the HeLa [^{32}P]DNA-protein complex reduced in the mixed samples (Table 2). This result indicated that the TBPs were still associated with the cellular DNA after treatment at 80°C in 2% SDS and that they do not exchange binding sites readily.

HeLa cell TBPs are enriched in the nuclear matrix fraction. Because the spacing between the TBPs was the same as the distance between attachment sites of DNA to the nuclear matrix (25, 48), we next investigated whether these proteins might be bound preferentially to DNA associated with the nuclear matrix. HeLa cell monolayers were labeled with $^{32}P_i$ for 65 to 70 h, and a nuclear matrix fraction was prepared by the method of Razin et al. (48). Isolated nuclei were digested with restriction enzymes or with low concentrations of micrococcal nuclease (1

TABLE 1. Spacing of TBPs along HeLa DNA

Sample DNA	DNA bound to filter (%)	Avg fragment size[a] (kb)	Avg Spacing[b] (Kb)
Control	46	31	50
*Sma*I digested	34	20	48
*Bam*HI digested	17	8.5	46
*Eco*RI digested	15	3.8	23
*Hae*III digested	14	0.5	35

[a] Determined by analytical velocity sedimentation.
[b] Calculated from Poisson statistics assuming that one or more protein molecules were sufficient to bind any DNA segment to the filter where Spacing = (fragment size)/[−ln(1 − fraction bound)]. Average of all spacing, 40 ± 11 kb.

Unit/ml) and the nuclei were extracted with 2 M NaCl. DNA-protein complexes were purified on Sepharose 4B-CL columns from both the nuclear matrix fraction and the solubilized DNA fraction. The complexes were then electrophoresed on 1% agarose gels before and after pronase treatment. The majority of the ^{32}P radioactivity in the nuclear matrix-bound DNA (Fig. 4, lane 1) was retained at the origin before proteolysis, but it did enter the gel after pronase treatment (lane 2). Conversely, the soluble DNA fraction had very little ^{32}P radioactivity remaining at the origin (lane 3), and the electrophoretic mobility of this DNA was not affected by pronase treatment (lane 4). The fraction of radioactivity in the nuclear matrix-bound DNA retained at the origin varied from 20 to 80% in different preparations, again reflecting the average fragment size of the DNA sample before chromatography. However, in all cases only the matrix-bound DNA fraction which remained after 2 M NaCl extraction had radioactivity retained at the gel origin which could be released into the gel by pronase treatment. These observations clearly indicated that the TBPs were highly enriched in the nuclear matrix fraction of the nucleus.

Association of the TBPs with DNA at nuclear matrix attachment sites. Because the TBPs were bound preferentially to DNA associated with the nuclear matrix, we were interested to see whether they might be directly involved in binding DNA to the nuclear matrix. Unfortunately, preparation of DNA-protein complexes by exclusion chromatography requires pieces of DNA large enough to chromatograph in the void volume, and this method cannot be used to probe closer to the nuclear matrix attachment sites than about 5 kb. We therefore exploited the unusual properties of the TBP-DNA complex (exclusion from agarose gels, filter binding, and resistance to protease digestion [see below]) to probe within 100 to 200 nucleotides of the nuclear matrix attachment sites. Identical samples of HeLa cells were labeled with $^{32}P_i$ for 24 h or labeled with [^{3}H]thymidine for 5 h; 1.3×10^7 cells from each sample were then harvested, and nuclei were isolated from the ^{32}P- and ^3H-labeled cells separately. The nuclei were treated with micrococcal nuclease (20 μg/ml) for 15 min at 37°C and extracted with 2 M NaCl. The nuclear matrix and solubilized DNA fractions were then isolated on sucrose-glycerol step gradients (48) and treated with RNase. In both samples the bulk DNA was digested to predominantly "nucleosome-size" pieces, and only 2% of the [^{32}P]DNA and 3% of the [^{3}H]DNA was found in the nuclear matrix-bound fraction. These samples were dialyzed overnight in TE, digested for 2 h at 37°C in 0.5% SDS and 0.6 mg of pronase per ml, and then assayed for the presence of TBPs by the filter-binding assay. (The protease conditions used do not digest the TBPs present in these DNA-protein complexes; see below.) The soluble DNA was essentially

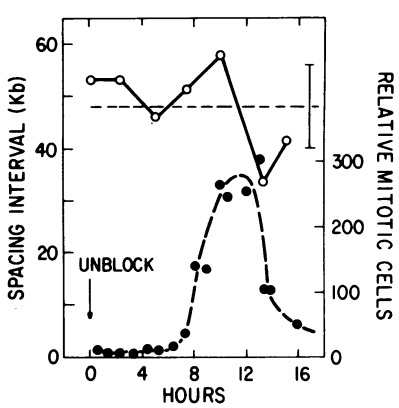

FIG. 3. Spacing of the TBPs on HeLa DNA through the cell cycle. As described in the text, ^{32}P-labeled HeLa DNA-protein complex was isolated from synchronized cells at the indicated times after release from thymidine blockage (56). These samples were subjected to agarose gel electrophoresis, and the protein spacing interval (○) was calculated using the fraction of ^{32}P radioactivity remaining at the origin and the average molecular weight of the DNA, as described in Table 1. The dashed line shows that the average spacing interval was 48 kb; the vertical bars indicate the standard error of the mean (±8 kb). Relative numbers of mitotic cells (●) were determined by counting metaphase cells in 10 random fields examined by phase-contrast microscopy.

TABLE 2. Exchangeability of TBPs on HeLa DNA after heating at 80°C in SDS

Sample	Pronase treatment	DNA bound to filter (%)	
		^3H	^{32}P
HeLa [^{3}H]DNA[a]	−	5.6	
	+	2.4	
HeLa [^{32}P]DNA-TBP complex[b]	−		9.8
	+		1.4
HeLa [^{32}P]DNA-TBP complex + HeLa [^{3}H]DNA[c]	−	4.2	10.0
	+	2.4	1.3

[a] Average size of DNA, 20,000 bp. Even though this DNA sample was phenol extracted many times, over 5% of the material was retained on the filter in the absence of protease treatment. Less than 1% of an adenovirus DNA sample was retained on the filter after similar treatment. This observation further indicates the difficulty in preparing truly protein-free HeLa cell DNA.

[b] Average size of DNA, 2,100 bp.

[c] The sample contained equal amounts (~30 μg) of [^{3}H]- and [^{32}P]DNA.

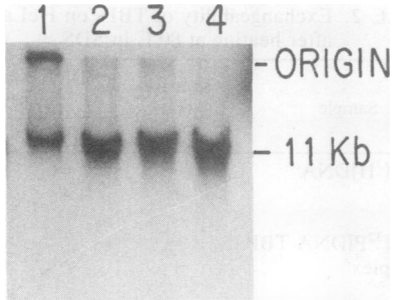

FIG. 4. Agarose gel electrophoresis of nuclear matrix-bound HeLa DNA-protein complex. Lane 1, DNA-protein complex, isolated from the HeLa nuclear matrix fraction, electrophoresed on a 1.0% agarose gel without proteolysis; lane 2, same DNA-protein complex after treatment with pronase (1 mg/ml) for 1 h at 37°C; lane 3, DNA solubilized by 2 M NaCl (see text) and electrophoresed before pronase treatment; lane 4, soluble DNA fraction after treatment with pronase (1 mg/ml) for 1 h at 37°C.

protein free, but the nuclear matrix-bound sample still showed the presence of the TBPs (Table 3). To eliminate the possibility that there were components in the nuclear matrix samples that bind DNA nonspecifically to the filter, the soluble [^{3}H]DNA and nuclear matrix [^{32}P]DNA were reciprocally mixed, and these mixtures were again assayed by filter binding. In the mixtures, only the nuclear matrix-bound DNA showed binding to the filters.

A similar experiment was done with ^{32}P-labeled cells, but in this case the nuclear matrix and soluble DNA fractions were assayed for TBPs with the agarose gel exclusion assay. When the intracellular DNA was digested to an average size of 140 bp (Fig. 5), essentially all of the soluble DNA (lane 2) was protein-free. In contrast, the majority of the DNA derived from the nuclear matrix fraction was retained at the gel origin (Fig. 5, lane 1). Thus, the TBPs are still bound to the cellular DNA present in the nuclear matrix fraction, even after the DNA has been reduced to an average size of only 140 bp. This observation strongly suggests that the TBPs are intimately involved in nuclear matrix-DNA interaction, and we suggest that these proteins anchor the cellular DNA to the matrix structure.

Identification of the TBPs. To identify the TBPs, nuclear matrix was prepared from 5×10^8 to 10^9 HeLa cells (48), with care taken to limit nuclease digestion so that the average DNA size was 5 to 15 kb. DNA-protein complexes were then isolated by exclusion chromatography on Sepharose 4B-CL (400-ml bed volume) by the method of Harter et al. (21). Samples were concentrated by ethanol precipitation, boiled for 2 min in 2% SDS, and then electrophoresed on 15% SDS-polyacrylamide microslab gels (32, 55). Two polypeptides of 55 and 66 kd were seen by Coomassie blue staining (Fig. 6A, lane 3). Samples were also digested with micrococcal nuclease (lane 2) or DNase I (not shown), but no additional protein bands were seen which were not present in the nucleases (protein bands present in the micrococcal nuclease preparation are marked by arrowheads). The 66- and 55-kd proteins were usually found in roughly equimolar amounts, suggesting the possibility that they form a specific complex when bound to the HeLa DNA.

The molecular weights of the HeLa TBPs were also compared with other proteins of the nuclear matrix. HeLa cell nuclear matrix was prepared as described by Lebkowski and Laemmli (30), and the protein components were separated by polyacrylamide gel electrophoresis (Fig. 6B, lane 3). As previously reported by others (1, 12, 23, 49, 59) the HeLa cell matrix contains seven major proteins with molecular weights between 45 and 70 kd. The 66-kd TBP, which in this and other experiments is often resolved as a doublet (Fig. 6B, lane 2), does not appear to have an electrophoretic mobility equivalent to any of the major proteins seen in a standard matrix preparation. Although the 55-kd TBP does comigrate with a major nuclear matrix protein, we have demonstrated that the matrix protein is predominantly vimentin (19) and that vimentin and the 55-kd TBP have different peptide maps when treated with V8 protease (data not shown). A detailed comparison of the peptide maps of the TBPs and the major nuclear matrix proteins is in progress and will be presented elsewhere. These observations would seem to indicate that the TBPs are minor constitutents of the nuclear matrix. However, for reasons enumerated below, we feel that the

TABLE 3. Association of TBPs with DNA at nuclear matrix attachment sites

Expt	DNA bound to filter (%)	
	^{3}H	^{32}P
[^{32}P]DNA		
Soluble		2
Matrix bound		63
[^{3}H]		
Soluble	1	
Matrix bound	47	
Mixtures		
Soluble [^{3}H]DNA+ matrix-bound [^{32}P]DNA	0.3	66
Matrix-bound [^{3}H]DNA + soluble [^{32}P]DNA	40	2

TBPs are generally underrepresented in the pool of nuclear matrix proteins.

The molecular weights of the HeLa cell TBPs were the same as those reported by D. Werner and his co-workers for the proteins found tightly bound to DNA extracted from mammalian, plant, and bacterial cells (7, 28, 54, 60–63). To determine whether these proteins were the same as the TBPs present in the nuclear-matrix fraction, we prepared DNA-protein complexes from HeLa cells by using the method of Werner et al. (63). The polypeptide pattern of this sample was identical by SDS gel electrophoresis to that seen with a TBP fraction prepared by exclusion chromatography (Fig. 7A, lanes 1 and 2). In this silver-stained gel several additional protein bands were observed. It is unclear at this time whether those bands represent proteins more weakly bound to cellular DNA or proteolytic

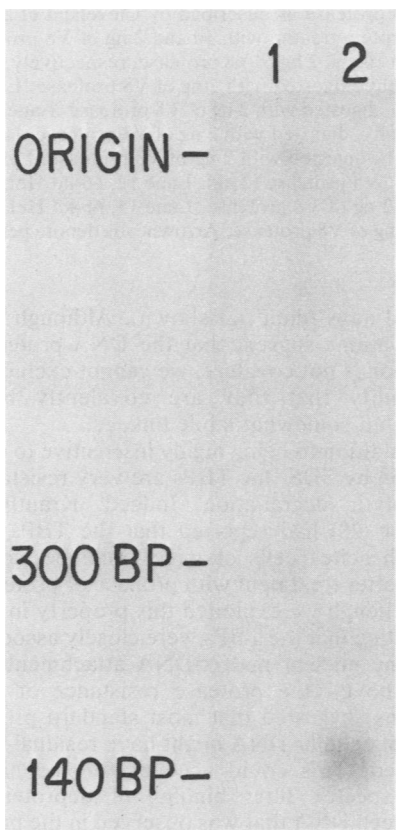

FIG. 5. Location of HeLa TBPs on the nuclear matrix-DNA attachment sites. HeLa nuclear matrix and solubilized DNA were isolated after extensive digestion of nuclei with micrococcal nuclease. Each sample was digested with pronase (0.6 mg/ml) in the presence of SDS and loaded directly onto a 2% agarose gel. Lane 1, Nuclear matrix fraction; lane 2, soluble DNA fraction.

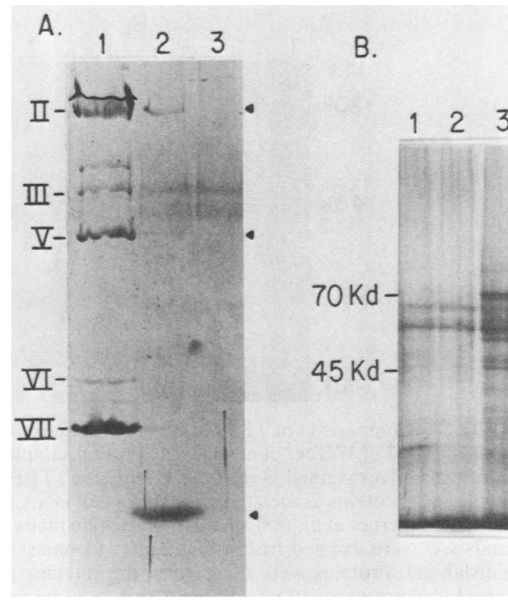

FIG. 6. Identification of the proteins in the HeLa DNA-protein complex. (A) HeLa DNA-protein complex was ethanol precipitated, boiled in 2% SDS, electrophoresed on 15% SDS-polyacrylamide slab gels, and stained with Coomassie blue R-280. Lane 1, Adenovirus type 2 virion protein markers: II, 120 kd; III, 62 kd; V, 48.5 kd; VI, 24 kd; VII, 18.5 kd. Lane 2, HeLa DNA-protein complex after digestion with micrococcal nuclease (10 mg/ml) for 3 h at 37°C; bands with arrowheads correspond to proteins present in the micrococcal nuclease. Lane 3, HeLa DNA-protein complex without nuclease treatment. (B) Comparison of the proteins tightly bound to the DNA of *E. coli* (lane 1) and HeLa cells (lane 2) with those of the HeLa cell nuclear matrix (lane 3).

fragments of the two TBPs. The 55- and 66-kd protein bands in each preparation were then cut out and compared by partial peptide analysis (8) after treatment with V8 protease. The V8 protease peptide maps of the two 66-kd proteins were identical (Fig. 7B, lanes 12 and 13), as were those of the 55-kd proteins (lanes 6 and 7). In addition, the 55- and 66-kd proteins appear to be related to each other, as they share several common polypeptides (compare lanes 6 and 7 with lane 9, Fig. 7B). The partial peptide map of a 55-kd TBP isolated from SV80 cells, a simian virus 40-transformed human cell line (lane 8), also exhibits homology to the corresponding proteins from HeLa cells. However, neither of the TBPs appears to be related to the 50- or 55-kd prekeratin proteins (lanes 2–5) or to β-tubulin (lane 10). We therefore conclude that the TBPs reported here and those reported by Werner and his co-workers (7, 28, 54, 60–63) are the same proteins. Furthermore, since Werner et al. have shown that antisera against TBPs of rat cells will

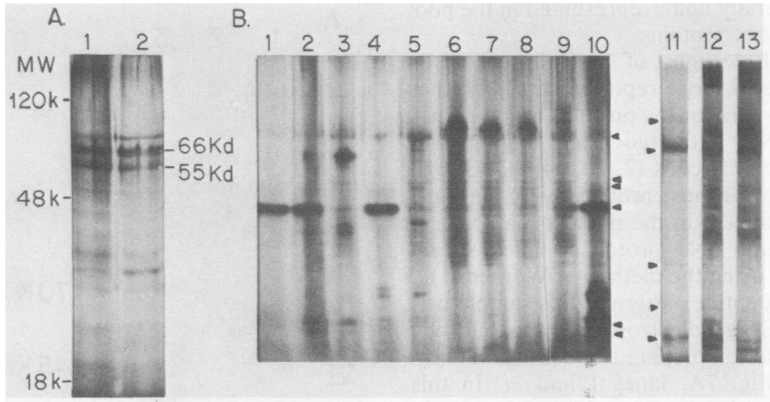

FIG. 7. Comparison of TBPs isolated by SDS exclusion chromatography with TBPs isolated from HeLa cells by the method of Werner et al. (63). (A) Protein samples were run on a 10% polyacrylamide-SDS minislab gel (29, 32, 55) and silver stained (36). Lane 1; HeLa cell TBPs isolated by SDS-Sepharose 4B-CL column chromatography; lane 2, proteins associated with HeLa cell DNA after cell lysis in alkaline SDS and phenol extraction by the method of Werner et al. (63). (B) Partial peptide maps of proteins tightly bound to HeLa DNA. The 55- and 66-kd bands (A) were excised from the gel after Coomassie blue staining, and the gel slices were applied to a 12% minislab gel. Proteins were digested in the stacking gel with V8 protease as described by Cleveland et al. (8). Lane 1, V8 protease (10 ng). Lanes 2 and 3, 50-kd prekeratin protein treated with 10 and 2 ng of V8 protease, respectively. Lanes 4 and 5, 55-kd prekeratin protein treated with 10 and 2 ng of V8 protease, respectively. Lane 6, 55-kd HeLa cell TBP isolated by SDS-exclusion chromatography, digested with 2 ng of V8 protease. Lane 7, 55-kd HeLa cell TBP isolated by the method of Werner et al. (63), digested with 2 ng of V8 protease. Lane 8, 55-kd TBP isolated from SV80 cells by SDS-exclusion chromatography, digested with 2 ng of V8 protease. Lane 9, 66-kd HeLa cell TBP isolated by the method of Werner et al. (63), digested with 2 ng of V8 protease. Lane 10, HeLa cell β-tubulin digested with 10 ng of V8 protease. Lane 11, V8 protease (2 ng). Lane 12, 66-kd HeLa cell TBP isolated by the method of Werner et al. (63), digested with 2 ng of V8 protease. Lane 13, 66-kd HeLa cell TBP isolated by SDS exclusion chromatography, digested with 2 ng of V8 protease. Arrowheads denote peptides present in the V8 protease sample.

immunologically react with the TBPs associated with DNA from eucaryotic, procaryotic, and plant cells, it is apparent that the two matrix-associated proteins are very highly conserved. Indeed, the TBPs purified from *Escherichia coli* cells (Fig. 6B, lane 1) and HeLa cells (lane 2) exhibited the same peptide pattern when analyzed by SDS-polyacrylamide gel electrophoresis. Some potential implications of these observations are presented below.

Additional characteristics of the HeLa DNA-protein complex. Although the TBPs were not dissociated from cellular DNA by heating at 80°C in 2% SDS, by phenol extraction, or by treatment for 30 min with 0.1 M NaOH, they did not appear to be covalently bound to the DNA. If the DNA-protein complex was boiled in the presence of SDS, the proteins were released and they migrated in an SDS-polyacrylamide gel as free proteins (Fig. 6A, lane 3). Similarly, the DNA component of the complex would migrate into an agarose gel after the complex had been boiled in the presence or absence of SDS (data not shown). Furthermore, if the DNA-protein complex was stored at 4°C, its ability to bind glass-fiber filters decreased with increasing storage time; filter-binding capacity was almost completely abolished after storage at 4°C for several days (data not shown). Although these observations suggest that the DNA-protein interaction is not covalent, we cannot exclude the possibility that they are covalently bound through a somewhat labile linkage.

In addition to being highly insensitive to denaturation by SDS, the TBPs are very resistant to proteolytic degradation. Indeed, Krauth and Werner (28) had reported that the TBPs from Ehrlich acites cells of mice could be isolated intact after treatment with pronase or proteinase K. Although we exploited this property in demonstrating that the TBPs were closely associated with the nuclear matrix-DNA attachment sites (see above), the protease resistance of these proteins suggested that most standard preparations of cellular DNA might have residual TBPs attached. This could account for the higher-than-expected filter binding of deproteinized HeLa cell DNA that was observed in the protein exchange experiment (Table 2). To test this possibility, several different preparations of HeLa DNA were made according to the procedure of Pearson and Hanawalt (44). Nuclei (^{32}P- or ^{3}H-labeled) were incubated in 0.5% SDS with 1 mg of pronase per ml for 2.5 to 20 h at 37°C; the residual material was extracted several times with phenol, and the aqueous phase was ethanol

precipitated. Each sample was then assayed for the presence of DNA-protein complexes by the filter-binding assay. A portion of each DNA sample retained the filter-binding characteristics of the DNA-protein complex (Table 4). The average protein spacing on these DNA samples varied from 32 kb (indicating that essentially none of the TBPs were removed) to over 400 kb (indicating that about 90% of the TBPs were released from the DNA); the extent of deproteinization was not, however, directly proportional to the time of protease treatment. The variability in the filter binding in these different DNA samples is most likely a direct reflection of the extent to which the TBPs are protease digested, as it is not an assay artifact. When the filtrate of a filter-binding assay was passed through a second filter, the amount of DNA retained on the second filter (1 to 1.5%) was similar to that observed with protein-free adenovirus DNA (Table 4, experiments 6A and 6B). It is apparent, therefore, that the complete removal of the TBPs from cellular DNA requires fairly drastic treatments.

The HeLa cell TBPs were also found to be extremely insoluble, highly hydrophobic, and difficult to label in vivo with [^{35}S]methionine. The DNA-protein complex also stuck to glass surfaces, although the presence of SDS in the sample buffers minimized this interaction. This detergent had to be present in all solutions to ensure optimum yields of the DNA-protein complex. In the absence of detergent the complex precipitated, and these precipitates were difficult to resolubilize, even after boiling in SDS. The general insolubility of the TBPs was particularly apparent once they were dissociated from their DNA binding sites. Although the general insolubility of these two proteins made it difficult to directly assess their abundance in the nucleus relative to the other matrix proteins, on the basis of the spacing distribution on the DNA (~40 kb) and assuming one molecule of each polypeptide per binding site, we estimate that ~250,000 molecules of each protein are present per HeLa cell nucleus. This copy number would mean that they are both relatively abundant nuclear proteins; however, because of their insolubility, they appear to be underrepresented in the pool of nuclear matrix proteins. Furthermore, although other nuclear matrix proteins are readily labeled in vivo with [^{35}S]methionine, bands corresponding to the TBPs could not be seen on autoradiographic exposures, even when the TBP bands were clearly visible by Coomassie blue staining of the gel (data not shown). This observation suggests either that the HeLa TBPs contain little methionine or that they have a very low turnover rate in the cell.

The sequence complexity of the DNA that interacts with nuclear matrix proteins has been reported to be highly repetitive (5, 27, 33), middle repetitive (48), or random unique (41). These variable results may be a reflection of the hydrophobicity of the TBPs and the size of the DNA fragments to which they are attached. Krauth and Werner (28) reported that their DNA-protein complexes remained in the aqueous layer during phenol extractions; however, their proteins were bound to large pieces of DNA. Since the precise identity of DNA sequences to which the TBPs are bound would require the isolation of TBP-DNA complexes in which the DNA fragments were small, we assessed the effect of phenol extraction on the recovery of the TBP-DNA complex with short DNA fragments. HeLa cells were labeled with $^{32}P_i$, and the nuclear matrix and soluble DNA fractions were isolated on sucrose-glycerol step gradients after extensive digestion with micrococcal nuclease. In this preparation 2% of the DNA was matrix bound. At this point deproteinized HeLa [^{3}H]DNA was added to each sample as a monitor for DNA recovery. Portions of the matrix-bound DNA, soluble DNA, and a mixture (containing an equal amount of ^{32}P from each DNA sample) were then phenol extracted and ethanol precipitated. The recovery of marker [^{3}H]DNA was quantitative throughout the extraction, and equal quantities (by [^{3}H]DNA recovery) of the various samples were run on 2% agarose gels (Fig. 8). The soluble DNA consisted predominantly of fragments 140 and 300 bp in length (Fig. 8, lane 1A). These were recovered essentially quantitatively after phenol extraction and ethanol precipitation (lanes 1B and C). However, the nuclear matrix-bound DNA sample, which consisted mainly of TBP-DNA complexes (lane 2A), remained at the gel origin. These DNA fragments were lost preferentially during phenol extraction (lane 2B). When the samples were mixed (lanes 3A, B, and C), the TBP-DNA complex was depleted from the mixture after phenol extraction (lane 3B). Thus it

TABLE 4. Residual TBPs found on HeLa cell DNA after pronase treatment, phenol extraction, and ethanol precipitation

Expt	Avg mol wt of DNA (kb)	DNA bound to filter (%)	Avg spacing (kb)
1	2	6.0	32
2	2	2.0	99
3	20	26.4	65
4	20	4.7	415
5	3	4.0	73
6A	12	8.0	143
6B[a]	12	1.5	

[a] Filter from experiment 6A.

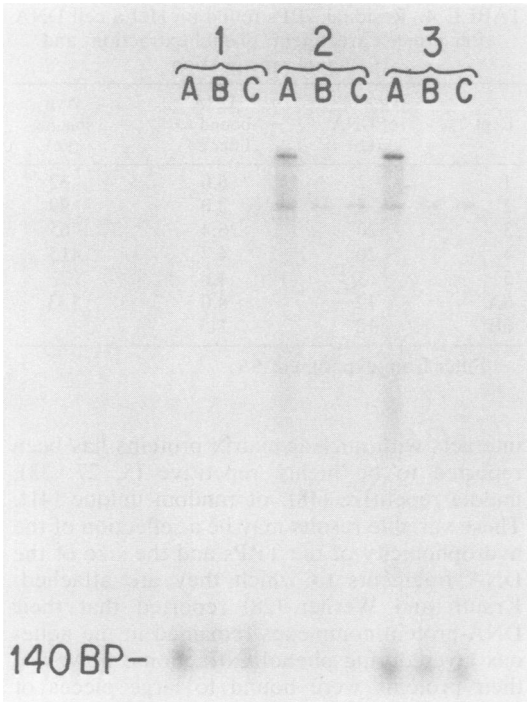

FIG. 8. DNA-TBP complexes were selectively removed from the aqueous layer during phenol extraction when the size of the DNA fragment is small. HeLa cell nuclear matrix DNA-protein complex and soluble DNA were isolated after extensive digestion of nuclei with micrococcal nuclease. Each sample was treated with pronase (1 mg/ml) in the presence of SDS. Sample 1, Soluble DNA; sample 2, nuclear matrix DNA-protein complex fraction; sample 3, mixed soluble DNA and nuclear matrix fraction. For each sample: Lane A, sample loaded directly on gel after SDS-pronase treatment; lane B, aqueous layer after phenol extraction; lane C, aqueous layer after phenol extraction and ethanol precipitation.

appears that the characteristics of the DNA-protein complexes from HeLa cells depend largely on the size of DNA to which the protein is bound; large DNA pieces (greater than several kb) tend to carry the proteins into the aqueous phase, but as the size of the DNA is reduced, the complex shows more characteristics of the TBPs, i.e., insolubility, loss in phenol extractions, hydrophobicity, and aggregation. We suggest that the conflicting results on the sequence complexity of the residual DNA associated with the nuclear matrix may in part be due to these properties of the DNA-protein complex.

DISCUSSION

A considerable body of evidence indicates that the genome of eucaryotic cells is organized into domains of supercoiled DNA that vary in size from 30 to 90 kb. Relaxation of the supercoiling in one domain does not affect the superhelicity of the adjacent domains (4, 12, 22). Metaphase chromosomes that are extracted with 2 M NaCl and spread for electron microscopy exhibit these DNA domains as loops of DNA radiating from a protein scaffold (43) or as rosettes of DNA (10, 40). During interphase the DNA is attached to the nuclear matrix (2, 52) at sites that are spaced ~40 to 60 kb apart (25, 48). This DNA-protein attachment interval is the same in both interphase chromatin and metaphase chromosomes, suggesting that the protein-DNA attachment sites are also unchanged throughout the cell cycle (48). Because the nuclear matrix is increasingly being implicated as a functional component in essential nuclear processes such as DNA replication and gene transcription (as reviewed in references 2, 9, 26, and 52) a detailed understanding of the nature of the DNA-matrix interaction is of considerable interest.

Here we report the isolation of a DNA-protein complex from HeLa cells by procedures originally developed to isolate the DNA-terminal protein complex of intranuclear adenovirus DNA (50). The HeLa DNA-protein complex contains two protein species, 55 and 66 kd in size, which appear to be very tightly, but not covalently, bound to the cellular DNA. These TBPs are distributed over the DNA with an average spacing of about 40 kb, and this spacing remains essentially constant throughout the cell cycle. We have also shown that these proteins are specifically enriched in the nuclear matrix fraction and that they are bound to the small residues of DNA which remain attached to the nuclear matrix after extensive nuclease digestion. Collectively, our observations suggest that these proteins participate in the attachment of cellular DNA to the nuclear matrix structure.

Stable protein-DNA complexes have been isolated from a wide variety of cell types, and many of these have been shown to contain proteins with molecular weights similar to those we have observed for the HeLa cell TBPs. For example, Andoh and co-workers (24, 37) isolated a protein-DNA complex from mouse carcinoma cells, after gentle cell lyses in SDS, which contained proteins of 55 and 70 kd. Plagens (46) reported that polytene chromosomes from *Chironomus tentans* retained proteins of 50 and 70 kd after extensive extraction with 2 M NaCl. These proteins were also associated with *Chironomus* DNA after protease treatment (20). Bekhor and co-workers (3, 39) isolated a DNA fraction from human placental cell chromatin, after extraction with 2 M NaCl, which was highly enriched in active gene sequences and contained TBPs. This DNA-protein complex was highly insoluble and could be further purified by cen-

trifugation in low-salt buffers; insolubility in low salt is a characteristic of the HeLa DNA-TBP complex as well. Matsui et al. (J. Cell Biol. **91**:60a, 1981) prepared histone-depleted metaphase chromosomes from Chinese hamster cells and found that proteins of 55 and 66 kd were major constituents of the residual chromosome scaffold. Finally, Razin et al. (47) have reported that proteins of ~52 and 60 kd are tightly bound to large DNA fragments associated with the nuclear matrix fraction of cells.

Werner and associates (7, 28, 54, 60–63) have shown that two proteins, reported as 54 and 68 kd, are found tightly bound to the DNA of many cell types (including those of mammals, plants, and bacteria), even after the DNA is treated with alkali, detergent, and proteases. Furthermore, Werner et al. (62) have shown that antibodies raised to the TBPs from mouse cells will immunologically cross-react with the TBPs associated with *E. coli* DNA. In this report we demonstrated that the 55- and 66-kd proteins that are bound to the residual DNA present in the HeLa cell nuclear matrix are identical, by peptide map analysis, to the TBPs isolated from HeLa cells by the extraction method of Werner's group. We also showed that the TBPs from HeLa cells comigrate on polyacrylamide gels with the TBPs extracted from *E. coli* (Figure 6B). (Indeed, the peptide maps of the TBPs from both cell types appear to be identical after treatment with V8 protease [data not shown].)

The observations described above indicate that the 55- and 66-kd proteins that we find at (or near) the DNA-nuclear matrix attachment sites in HeLa cells are the human cell representatives of a family of highly conserved proteins. Furthermore, since the HeLa cell TBPs are clearly associated with the nuclear matrix and interact with cellular DNA with a spacing distance of ~40 kb, we suggest that in eucaryotic cells these proteins function to anchor the DNA to the nuclear matrix (or chromosome scaffolds) and thus participate in organizing the genome into structural and functional domains. By analogy, we argue that the TBP-DNA interactions in bacterial cells are most likely involved in maintaining the higher order structure of the bacterial nucleoid (as reviewed in reference 45).

The interpretation of the data presented here is consistent with the general model of chromosome organization and DNA replication proposed by Dingman (17). He suggested that static nuclear matrix-DNA interactions would occur at attachment sites near DNA replication origins, thereby delineating the boundaries of replicon units, whereas dynamic nuclear matrix-DNA interactions would occur at DNA replication forks. We suggest that the DNA sequence(s) which interact(s) with the HeLa TBPs represents such static attachment sites. Although the physical characteristics of the TBP-DNA complex (tenacious binding and an average DNA spacing distance of ~40 kb) support the DNA replication scheme of Dingman, they are also fully compatible with the basic model of chromosome structure put forward by Marsden and Laemmli (31).

In all of the preparations of TBP-DNA complexes, we have observed DNA fragments that are bound to the nuclear matrix in 2 M NaCl but are not associated with the TBPs (see Fig. 4, 5, and 8). These may represent DNA segments that are bound to the nuclear matrix via interactions with proteins at replication forks or in transcription complexes, as is proposed by Dingman (17). The presence in nuclear matrix preparations of DNA that is either TBP bound or TBP free may also help to explain the controversy concerning the complexity of the DNA sequences that are actually bound at the nuclear matrix attachment sites. Although there is considerable evidence that specific genes are located nonrandomly with respect to the nuclear matrix (11, 13, 38, 51), the sequence of the attachment region has been reported to be satellite DNA (5, 27, 33), middle-repetitive DNA (48), or random unique sequence DNA (42). We have shown that the proteins in the TBP-DNA complex are highly resistant to proteolysis and that different DNA preparations contain variable amounts of residual TBPs. If the DNA in the complex is large (as in the case when matrix DNA is analyzed with specific gene probes), the TBP-DNA complexes will have the basic characteristics of free DNA during phenol extractions. However, when the TBPs are attached to small DNA fragments, the DNA is partitioned into the phenol layer (see Fig. 8). Therefore, unless the conditions under which the matrix-bound DNA is isolated are carefully controlled and monitored, variable results can be expected. We are currently separating nuclear matrix-bound DNA into TBP-bound and TBP-free fractions for cloning and sequence analysis.

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