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Isolation of human DNA sequences that bind to nuclear factor I, a host protein involved in adenovirus DNA replication

(protein-mediated selection/origins of DNA replication/site-specific DNA binding)

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ABSTRACT **Nuclear factor I is a 47,000-dalton protein isolated from human HeLa cells that is required for the *in vitro* replication of adenovirus DNA. This protein was previously shown to bind specifically to nucleotides 17–48 of the left-hand terminus of cloned adenovirus serotype 5 DNA. An *in vitro* assay for DNA sequences that compete with adenovirus DNA for the binding of nuclear factor I has been developed. With this assay, we have shown specific binding of human DNA sequences to nuclear factor I. Using the DNA binding activity of nuclear factor I, we have isolated and cloned segments of human DNA that bind tightly to this protein. One nuclear factor I binding site is present about every 100,000 base pairs in the HeLa cell genome. The binding of these DNA molecules to nuclear factor I resembles the binding of cloned adenovirus DNA to the protein and is resistant to high ionic strength. The isolation of DNA sequences from HeLa cells that bind specifically to nuclear factor I suggests that this protein interacts with host DNA *in vivo*.**

Site-specific DNA binding proteins are essential for transcription (1–7), recombination (8–10), packaging (11), and replication (12–16). Most studies of site-specific DNA binding proteins in eukaryotes have used proteins or DNA molecules that are encoded by viruses such as simian virus 40, murine mammary tumor virus, and adenovirus (Ad). In studies on simian virus 40 (5) and murine mammary tumor virus transcription (4) and Ad DNA replication (16), proteins encoded by the host-cell genome have been shown to bind specifically to regions of viral DNA and thus regulate transcription and/or replication. Cellular DNA sequences that interact with these host proteins have not previously been described.

Nuclear factor I, a 47,000-dalton protein isolated from HeLa cells, stimulates the initiation of Ad DNA synthesis *in vitro* (17). This protein binds specifically to a 32-nucleotide region of cloned Ad serotype 5 (Ad5) DNA (16) at a site required for DNA synthesis (18). Only five proteins are required for the *in vitro* synthesis of full-length Ad DNA (17, 19, 20). Two proteins are host-encoded (nuclear factors I and II), while three proteins are virus-encoded [the precursor to the 5′-terminal protein (pTP), the Ad DNA polymerase, and the Ad DNA binding protein]. Ad DNA is a 35-kilobase (kb) linear molecule with a 55,000-dalton protein covalently attached to each 5′ terminus (21). The initiation of Ad DNA replication occurs at either terminus of the DNA molecule by the covalent attachment of dCMP to the pTP (22, 23). This pTP–dCMP complex is then elongated to full-length DNA with the displacement of a parental strand. Formation of the pTP–dCMP complex on Ad DNA requires the presence of Ad DNA polymerase, DNA binding protein, and pTP (24). In the presence of these viral proteins, nuclear factor I stimulates the initiation reaction about 10-fold (17). Nuclear factor II, which possesses type I DNA topoisomerase activity, is required only after substantial elongation of the DNA chains (25%–30% of full length) and is essential for the synthesis of full-length 35-kb Ad DNA (19, 20).

To better understand the role of nuclear factor I in host DNA metabolism, we have devised a strategy for the selection, isolation, and cloning of host DNA fragments that bind nuclear factor I. This strategy utilizes the nuclear factor I-mediated retention of DNA fragments on nitrocellulose filters and may be applicable to the isolation of DNA sequences recognized by other site-specific DNA binding proteins.

MATERIALS AND METHODS

Preparation of DNA. Plasmid pLA1 (25) was originally provided by F. Tamanoi and B. W. Stillman. pLA1 DNA was prepared by alkaline lysis followed by centrifugation to equilibrium in ethidium bromide/CsCl gradients (26). High molecular weight DNA from the nuclei of HeLa cells was prepared by spooling on a glass rod as described (27). Large-scale preparations of plasmid DNA grown in the DH-1 strain (28) of *Escherichia coli* were made by alkaline lysis and ethidium bromide/CsCl gradient centrifugation (26). Alkaline phosphatase-treated *Hin*dIII-digested pBR322 DNA was purchased from New England Biolabs.

Labeling of DNA. The 5′-labeled DNA was prepared by treatment of DNA with bacterial alkaline phosphatase (Worthington), T4 polynucleotide kinase (P-L Biochemicals), and [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) as described (26). The 3′-labeled DNA was prepared by treatment of DNA with restriction enzymes that generate 3′ recessed termini followed by repair of the termini with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) or the large fragment of *E. coli* DNA polymerase I (Boehringer Mannheim) and the appropriate [α-^{32}P]dNTPs (26). All restriction endonucleases were purchased from New England Biolabs and digestions were done as recommended by the manufacturer.

Purification of Nuclear Factor I. Nuclear factor I was purified from nuclear extracts of HeLa cells as described (17). Either the single-strand DNA cellulose fraction or the glycerol gradient fraction was used as indicated.

Nitrocellulose Binding Assay. Retention of labeled DNA fragments on nitrocellulose filters was as described by Nagata *et al.* (16) in reaction mixtures (50 μl) containing binding buffer [25 mM sodium Hepes buffer, pH 7.5/5 mM $MgCl_2$/4 mM dithiothreitol/bovine serum albumin (200 μg/ml) (Pentax fraction V)], 0.5–10 ng of nuclear factor I, and 50–150 mM NaCl as indicated in the figure legends. ^{32}P-labeled DNA was measured by Cerenkov radiation or by liquid scin-

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Abbreviations: Ad, adenovirus; Ad5, adenovirus serotype 5; pTP, precursor to the 55,000-dalton protein on the 5′ terminus of adenovirus DNA; FIB, nuclear factor I binding; bp, base pair(s); kb, kilobase(s); RFI, covalently closed circular duplex superhelical DNA.

tillation counting. In some experiments, DNA fragments were eluted from the nitrocellulose filters as described (16) and analyzed by electrophoresis on 0.8% or 2% agarose gels in Tris acetate buffer (50 mM Tris acetate, pH 7.5/2 mM EDTA). Gels were dried and subjected to autoradiography with x-ray film (Kodak X-AR).

DNA Competition Assay. The ability of various DNAs to compete with the Ad DNA sequences present in the plasmid pLA1 for binding to nuclear factor I was determined as follows: unlabeled competitor DNA (0.05–1 μg) was incubated in binding buffer containing 150 mM NaCl with a 3′ ^{32}P-labeled 453-base-pair (bp) *Eco*RI/*Pvu* II fragment of pLA1 DNA. This labeled fragment contains the 32-nucleotide region that is bound specifically by nuclear factor I. Nuclear factor I (10–50 ng) was added, and the reaction mixture was incubated at 4°C for 20 min, filtered through a nitrocellulose filter (Millipore, HAWP), and washed as described (16). The amount of labeled pLA1 DNA retained on the nitrocellulose filter in the presence or absence of competitor DNA was determined by liquid scintillation counting.

Cloning of HeLa Cell DNA Fragments Bound by Nuclear Factor I. High molecular weight DNA from HeLa cells was digested to completion with *Hin*dIII restriction endonuclease. A portion of the DNA was labeled at the 5′ terminus with T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol). The 5′-labeled DNA was mixed with unlabeled DNA to a final specific activity of 680 cpm/ng, and the resulting mixture (0.6 μg) was incubated with or without 25 ng of nuclear factor I (glycerol gradient fraction) in binding buffer containing 150 mM NaCl at 4°C for 20 min. The samples were then filtered through nitrocellulose filters previously washed with alkali (29), the filters were washed with 2.5 ml of binding buffer lacking bovine serum albumin and containing 150 mM NaCl, and the amount of DNA retained on the filters was determined by Cerenkov radiation (≈0.2% and 4% of the input DNA was retained in the absence and presence of nuclear factor I, respectively). The DNA fragments were eluted from the filters by placing the filter discs (≈1.5-cm-diameter circles) in polypropylene Eppendorf centrifuge tubes (1.5 ml) containing 300 μl of 1 M ammonium acetate and by heating at 68°C for 1 hr. The 1 M ammonium acetate eluate was extracted once with phenol/chloroform concentrated to 50 μl by two sequential extractions with *sec*-butanol and extracted twice with water-saturated ether. *Hin*dIII-digested alkaline phosphatase-treated pBR322 DNA (0.1 μg) was added to each sample, the samples were incubated in a dry-ice/ethanol bath for 60 min, and the DNA was collected by centrifugation at 15,000 × *g* at 4°C for 15 min. The pellet was dried under vacuum and dissolved in 10 μl of H_2O. To ligate the HeLa DNA fragments to the vector, 2 μl of a ligation buffer (500 mM Tris·HCl, pH 7.5/100 mM $MgCl_2$/100 mM dithiothreitol/10 mM ATP) was added, the samples were brought to a volume of 19 μl with water, and 0.9 unit of T4 DNA ligase (Boehringer Mannheim) in 1 μl of storage buffer was added. After incubation at 16°C for 20 hr, 10 μl of the reaction mixture was used to transform *E. coli* strain DH-1 as described (28). Frozen competent cells were used for all transformations. After transformation, 100-μl aliquots of the cells were plated on LM agar plates (28) containing ampicillin (50 μg/ml). After 12–16 hr at 37°C, colonies were picked and placed in ordered arrays on LM agar plates containing either ampicillin (50 μg/ml) or tetracycline (17 μg/ml). Tetracycline-sensitive ampicillin-resistant clones were selected for further analysis. DNA was isolated from 1.5 ml of overnight cultures of the clones as described (26).

RESULTS

Competition Assay. Nuclear factor I binds specifically to nucleotides 17–48 of the left-hand end of Ad5 DNA present in the plasmid pLA1 (16). When this plasmid is digested with *Eco*RI, the center of the nuclear factor I binding (FIB) site is ≈30 nucleotides from one terminus. Nagata *et al.* (16) demonstrated that nuclear factor I can bind to the FIB site even when the DNA sequence is present near the middle of a long DNA molecule. However, this study did not determine whether nuclear factor I could bind a FIB site present in a covalently closed superhelical DNA molecule. A competition assay was developed to answer this question. A 453-bp *Eco*RI/*Pvu* II fragment of pLA1 DNA contains the FIB site and is specifically retained on nitrocellulose filters by nuclear factor I (16). When unlabeled *Eco*RI-digested pLA1 DNA was added to reactions containing nuclear factor I and this 453-nucleotide fragment of pLA1 DNA labeled at its 3′ terminus, a dose-dependent decrease in the retention of the 453-nucleotide fragment was observed (Fig. 1). pBR322 DNA was much less effective at inhibiting the nuclear factor I-mediated retention of the labeled 453-bp fragment. However, covalently closed superhelical pLA1 DNA prevented retention of the 453-nucleotide fragment to the same extent as did *Eco*RI-digested pLA1 DNA (Fig. 1). Thus, a DNA terminus is not required for the specific binding of nuclear factor I to a FIB site.

Since this competition assay provided a rapid method to identify DNAs that competed with the FIB site on pLA1 DNA, we examined the ability of several different DNAs to act as competitors. Neither the covalently closed circular duplex superhelical DNA (RFI) forms of φX174, M13mp8, pBR322, simian virus 40, bovine papilloma virus nor several plasmids that contained *Alu* I family (30) and *Kpn* I family (31) DNA sequences competed with the 453-bp fragment of pLA1 DNA for binding to nuclear factor I (data not shown). However, a *Hin*dIII digest of HeLa cell DNA prevented the nuclear factor I-mediated adsorption of the pLA1 453-nucleotide fragment to nitrocellulose filters (Fig. 2). In several experiments, *Hin*dIII-digested HeLa cell DNA competed about 1/10th as well on a weight basis as pLA1 DNA for binding to nuclear factor I (data not shown). Assuming that this competition is due to specific binding by FIB sites on the DNA, one FIB site is present every 50–100 kb in the HeLa cell genome. This estimate does not consider the small

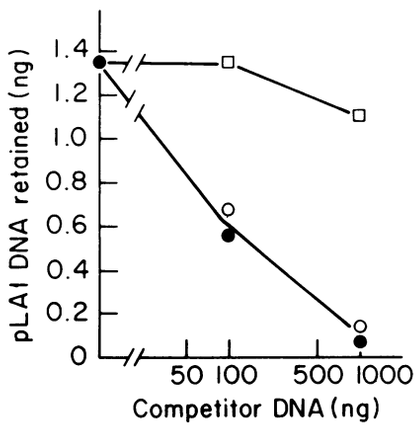

FIG. 1. Competition assay for FIB sites. The indicated amounts of competitor DNA were mixed with 3 ng of a 3′ ^{32}P-labeled 453-bp nucleotide *Eco*RI/*Pvu* II fragment of pLA1 DNA (1880 cpm/ng) in binding buffer containing 150 mM NaCl. Nuclear factor I was added (44 ng; single-strand DNA cellulose fraction), and the samples were incubated at 4°C for 20 min, filtered through nitrocellulose filters, and washed with 2.5 ml of binding buffer lacking bovine serum albumin and containing 150 mM NaCl. The filters were dried and radioactivity retained on the filter was determined by liquid scintillation counting. □, pBR322 RFI; ○, pLA1 RFI; ●, *Eco*RI-digested pLA1 linear duplex DNA.

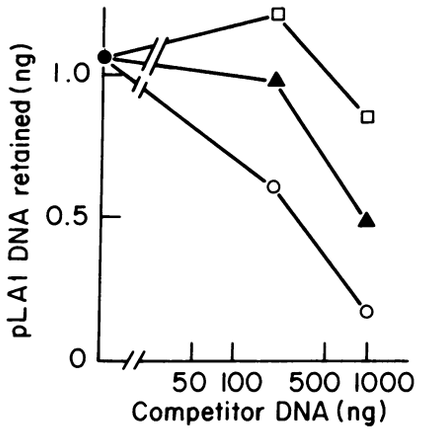

FIG. 2. Competition of HeLa DNA with pLA1 (Ad5) DNA. The indicated amounts of competitor DNA were mixed with 4 ng of 3′ ^{32}P-labeled pLA1 453-bp fragment (1880 cpm/ng), 44 ng of nuclear factor I (single-stranded DNA cellulose fraction) was added, and the samples were incubated, filtered, and washed as described in the legend to Fig. 1. □, pBR322 RFI; ▲, *Hin*dIII-digested HeLa cell DNA; ○, pLA1 RFI.

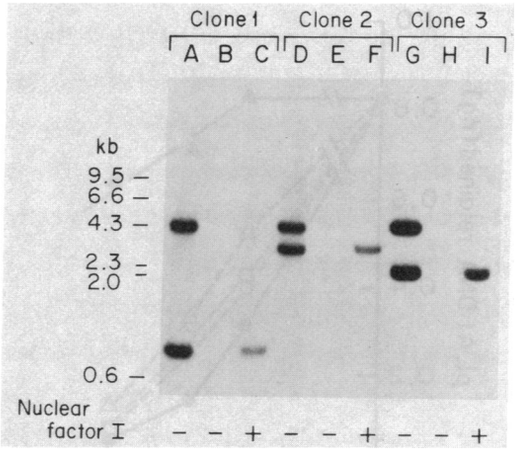

FIG. 3. Selective binding of HeLa cell DNA by nuclear factor I. 3′ ^{32}P-labeled *Hin*dIII digests of clones 1 (4.5 ng), 2 (3.6 ng), and 3 (9.0 ng) were incubated at 4°C for 20 min with or without 44 ng of nuclear factor I (single-strand DNA cellulose fraction) in binding buffer with 150 mM NaCl. The samples were filtered and washed, and the DNA fragments were eluted from the filter and analyzed by electrophoresis on a 0.8% agarose gel. Lanes A–C, clone 1; lanes D–F, clone 2; lanes G–I, clone 3. Lanes A, D, and G contained digests applied directly to the gel (unfiltered); lanes B, E, and H contained DNA retained on filters in the absence of nuclear factor I; lanes C, F, and I contained DNA retained on filters in the presence of nuclear factor I. The specific activities of clones 1, 2, and 3 were 4200 cpm per ng of DNA, 3800 cpm per ng of DNA, and 1500 cpm per ng of DNA, respectively.

amount of nonspecific binding of nuclear factor I by the large quantity (≤1 μg) of HeLa cell DNA used in the competition assay. Nonspecific binding would increase the apparent frequency of FIB sites in competitor DNA.

Cloning of HeLa DNA FIB Sites. Since *Hin*dIII-digested HeLa cell DNA competed with pLA1 DNA for binding of nuclear factor I, we devised a cloning strategy to select FIB sites from HeLa cell DNA. When plasmid DNAs from 48 colonies isolated by this selection procedure were screened for the presence of inserts by digestion of the DNAs with *Eco*RI, 10 contained inserts ranging in size from 700 to 6000 nucleotides. The remaining 38 may have contained inserts too small (<200 bp) to be easily detected by electrophoresis on agarose gels. When the 10 DNAs that contained inserts were tested for their ability to compete with pLA1 DNA for the binding of nuclear factor I, 3 were definitely positive, 5 were negative, and 2 were intermediate (data not shown).

The 3 DNAs that tested positive in the competition assay were digested with *Hin*dIII to release the insert DNA, labeled at their 3′ termini, and tested for binding to nuclear factor I. The three clones, FIB-1, -2, and -3, had inserts of ≈800, ≈3000, and ≈2200 bp, respectively. When the labeled molecules were incubated with nuclear factor I and filtered, only the insert fragments were retained on nitrocellulose (Fig. 3). The binding of the insert DNAs was via a specific interaction with nuclear factor I and was competed out by pLA1 DNA but not by pBR322 DNA (data not shown).

Characterization of HeLa DNA FIB Sites. The retention of the isolated insert DNAs by increasing amounts of nuclear factor I was similar to that seen for pLA1 DNA (Fig. 4). Cellular FIB sites 1 and 2 were bound by nuclear factor I in a similar manner to pLA1 DNA. At saturating levels of nuclear factor I, FIB-3 DNA was bound about one-half as well as pLA1, FIB-1, and FIB-2 DNA. The binding of nuclear factor I to FIB-1, -2, and -3 was resistant to high ionic strength (150 mM NaCl) as was the binding of the protein to pLA1 DNA (Figs. 3 and 4; unpublished data). In a competition assay, DNAs containing FIB sites 1, 2, and 3 competed as effectively as pLA1 DNA for binding to nuclear factor I (Fig. 5). Thus, FIB sites 1, 2, and 3, isolated from cellular DNA, show binding properties similar to those of the viral DNA sequence used to originally characterize nuclear factor I (16).

The structures of the cellular FIB sites were analyzed by restriction mapping (Fig. 6). Since the FIB site on Ad5 DNA was localized to a 32-nucleotide site bounded by nucleotides 17 and 48 on the Ad genome, it was anticipated that the cellular FIB sites would occupy only a small portion of each clone. We have currently localized the FIB sites to polynucleotides 600, 900, and 400 bp long on FIB sites 1, 2, and 3, respectively (Fig. 6, hatched bar). Further restriction site and sequence analysis should narrow these boundaries.

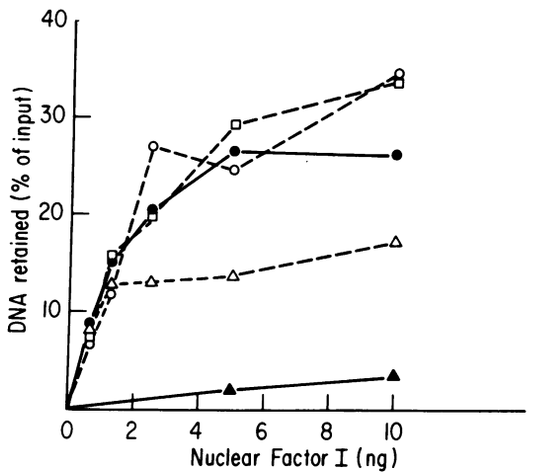

FIG. 4. Binding of various DNAs by nuclear factor I. 3′ ^{32}P-labeled DNA (8 fmol of linear molecules) was incubated at 4°C for 20 min with the indicated amount of nuclear factor I (glycerol gradient fraction) in binding buffer containing 150 mM NaCl. The sample was filtered and washed, and the DNA retained on the filter was measured by liquid scintillation counting. ●, 453-bp *Eco*RI/*Pvu* II fragment of pLA1 (Ad5) DNA (1080 cpm/fmol); ▲, *Hin*dIII-digested pBR322 (790 cpm/fmol); ○, 800-bp insert of clone 1, FIB-1 (410 cpm/fmol); □, 3000-bp insert of clone 2, FIB-2 (740 cpm/fmol); △, 2100-bp insert of clone 3, FIB-3 (500 cpm/fmol). FIB-1, -2, and -3 contained *Hin*dIII termini. The amount of DNA retained on the filters in the absence of nuclear factor was 2.6% for the six DNAs and was subtracted from the values shown.

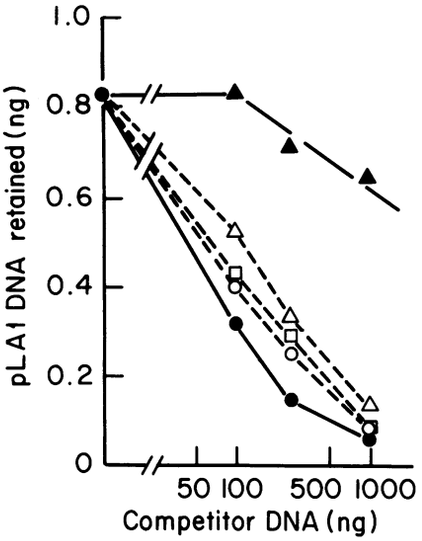

FIG. 5. Competition by FIB site clones for nuclear factor I. The indicated amounts of competitor DNA were mixed with 4 ng of 3' ^{32}P-labeled pLA1 453-bp fragment (2600 cpm/ng) as described in the legend to Fig. 1. Nuclear factor I (44 ng; single-strand DNA cellulose fraction) was added, and the samples were incubated, filtered, and washed. DNA retained on the nitrocellulose filters was determined by liquid scintillation counting. ●, pLA1 RFI; ▲, pBR322 RFI; ○, FIB-1 RFI; □, FIB-2 RFI; △, FIB-3 RFI.

DISCUSSION

Analysis of FIB Sites. The FIB site (nucleotides 17–48) on pLA1 DNA, in conjunction with nuclear factor I, is required for the initiation of Ad DNA synthesis *in vitro* (18). The effect of nuclear factor I on Ad DNA replication is probably mediated by its specific binding to this DNA sequence. One way to evaluate the DNA structures essential for nuclear factor I binding is to characterize the cellular nuclear factor I binding sites. This approach is potentially more fruitful than site-directed mutagenesis of the Ad5 FIB site, because it could elucidate the range of DNA sequences and/or secondary structures with which nuclear factor I interacts. For instance, the various factor Y binding sites present at different prokaryotic viral origins of lagging strand DNA replication share little if any sequence homology but may possess similar secondary structures (15, 32). Similarly, a preliminary Southern blot analysis has failed to detect any large degree of DNA sequence homology among the cellular and Ad5 FIB sites (unpublished data). Further analysis of the cellular FIB sites should determine what DNA sequences or possible DNA secondary structures are required for nuclear factor I binding to FIB sites. While testing clones isolated by the selection techniques described in *Materials and Methods*, one clone was identified that bound specifically to the nuclear factor I, but to only 1%–5% the extent of FIB sites 1, 2, and 3 (data not shown). Analysis of this clone and FIB-3 (which is bound less well than FIB-1 and FIB-2 DNA) may yield information on possible DNA sequences that modulate the binding of nuclear factor I to FIB sites.

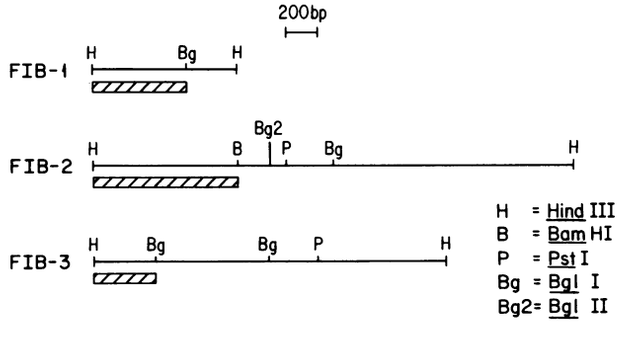

FIG. 6. Restriction maps of FIB site clones. Single and multiple enzyme digests were carried out by standard methods. Hatched bars indicate the location of the FIB sites on each clone. All three clones are oriented in pBR322 so that the FIB site is adjacent to the *Eco*RV site in pBR322.

Possible Functions of Cellular FIB Sites. Nuclear factor I was isolated by its ability to function in Ad DNA synthesis *in vitro*. The role of this protein in the host cell is unknown. The function of nuclear factor I in HeLa cells may be similar to its function in Ad DNA synthesis—i.e., the initiation of DNA replication. Conversely, it is possible that Ad, through evolution, has usurped the use of a protein originally designed for a role in transcription or recombination and adapted its function for use in DNA replication. The isolation of these cellular DNA sequences that bind to nuclear factor I should help elucidate the role of this protein in host DNA metabolism. For example, it is of interest to know whether the cellular FIB sites interact specifically with other host proteins presumably involved in DNA replication such as the HeLa cell DNA primase–DNA polymerase α complex (33) or DNA topoisomerases (34, 35).

From competition assays with labeled pLA1 DNA, the frequency of FIB sites in HeLa DNA was estimated at one per 50–100 kb (Fig. 2; unpublished data). During the cloning procedure, ≈4% of HeLa DNA fragments were retained on nitrocellulose filters in the presence of nuclear factor I. Thus, if all the FIB sites present in the genome were retained on the filter, a 25-fold enrichment was achieved. Since 30% (3 of 10) of the insert DNAs analyzed from the cloning protocol contained FIB sites, then the abundance of FIB sites in the unfractionated HeLa genome is 1.2% (0.3/25) of the DNA fragments. Since *Hin*dIII should cut the DNA every 4^6 bp (4096 bp; uncorrected for G+C content), then one FIB site is present approximately every 340 kb. This estimate is based on the complete retention of FIB sites by nuclear factor I. Under the binding conditions used in the cloning procedure (150 mM NaCl), only ≈30% of pLA1, and FIB-1, -2, and -3 DNA was retained by excess nuclear factor I (Fig. 4). Using this correction, it appears that one FIB site is present every 100 kb in the HeLa cell genome, an estimate in agreement with that obtained by the competition assay (Fig. 2). This frequency is similar to that of the families of long interspersed repeated sequences found in the human genome (36). However several cloned *Kpn* I family members (31) failed to compete with pLA1 DNA for binding to nuclear factor I (data not shown). Interestingly, a frequency of one site every 100 kb is also similar to the spacing of origins of replication in various mammalian cell lines (37).

Potential Uses for Protein-Mediated Cloning. The present study demonstrates the feasibility of isolation of cellular DNA sequences that bind tightly to a cellular protein. The enrichment of DNA sequences attained in two experiments was 25- to 100-fold. This level of enrichment is limited by the frequency of FIB sites in the human genome (one every 50–200 kb). For less abundant DNA sequences, sequential rounds of protein-mediated selection and transformation into *E. coli* should allow enrichments >10,000-fold. This approach may be used to select clones directly from a plasmid or cosmid library, thus eliminating any ligation steps after nitrocellulose filtration. This technique should be applicable to the isolation of cellular DNA sequences that bind to other known cell and virally encoded transcription (3–5) and replication factors (12, 14).

Note Added in Proof. Weideli *et al.* (38) used a cloning procedure similar to that described here to isolate DNA sequences that bind to protein DB-2 of *Drosophila*.

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