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Analysis of Multiple Forms of Nuclear Factor I in Human and Murine Cell Lines

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Nuclear factor I (NFI) is a group of related site-specific DNA-binding proteins that function in adenovirus DNA replication and cellular RNA metabolism. We have measured both the levels and forms of NFI that interact with a well-characterized 26-base-pair NFI-binding site. Five different NFI-DNA complexes were seen in HeLa nuclear extracts by using a gel mobility shift (GMS) assay. In addition, at least six forms of NFI were shown to cross-link directly to DNA by using a UV cross-linking assay. The distinct GMS complexes detected were composed of different subspecies of NFI polypeptides as assayed by UV cross-linking. Different murine cell lines possessed varying levels and forms of NFI binding activity, as judged by nitrocellulose filter binding and GMS assays. The growth state of NIH 3T3 cells affected both the types of NFI-DNA complexes seen in a GMS assay and the forms of the protein detected by UV cross-linking.

Nuclear factor I (NFI) was first isolated from HeLa cells as a host protein required for the initiation of adenovirus DNA replication in vitro (29). NFI mediates this effect by binding to a specific site within the adenovirus type 5 origin of replication (13, 30, 34, 36, 46). Analysis of this site and a number of other NFI-binding sites revealed a consensus sequence, TGGC/A(N)$_5$GCCAA, required for optimal binding (4, 9, 11). In addition, flanking sequences and the length and composition of the 5-base-pair spacer region can greatly modulate binding efficiency (9).

NFI-binding sites are present within the promoters of viral (14, 18, 42) and cellular (7, 19, 23, 38, 39) genes. Mutations of NFI-binding sites in the promoters of the hepatitis B virus S-antigen gene and the murine α2(I) collagen gene result in a loss of transcription in vivo, indicating a role for NFI in RNA synthesis (38, 42). Furthermore, the strength of NFI binding to sites in chimeric promoters correlated with stimulation of transcription in vitro and in vivo (12; P. Rebstein and R. M. Gronostajski, submitted for publication) Finally, it has been shown that NFI is probably identical to CCAAT-binding transcription factor (CTF), a protein implicated in the expression of a variety of genes (17, 19, 41).

Purification of NFI from HeLa cells has revealed a number of polypeptides, ranging in size from 52 to 66 kilodaltons (kDa), which constitute a family of NFI proteins (35). In addition, multiple mRNA species encoding NFI-family proteins have been detected from hamster (7), rat (32), porcine (27), and human (27, 41) sources.

We have used nitrocellulose filter binding, gel mobility shift (GMS) analysis, and UV cross-linking to assess the levels and forms of NFI present in various cell lines. Multiple specific protein-DNA complexes have been detected in nuclear extracts. In addition, cell-line-specific differences in both the forms and the absolute amounts of NFI binding activity were observed.

MATERIALS AND METHODS

Synthetic oligonucleotides. Oligonucleotides were made on an Applied Biosystems model 280A DNA synthesizer. Oligonucleotides FIB-2.6 (AGGTCTGGCTTTGGGCCAAGA GCCGC) and FIB-2.6C2 (AGGTCTCGCTTTGGGCCAAG AGCCGC) were made duplex by hybridization of a primer (GCGGCTCTTGGC), followed by extension using the large fragment of DNA polymerase I as previously described (9). When labeled with [α-^{32}P]dCTP, the initial specific activity of the duplex fragments was about 10,000 cpm/fmol.

Competitor DNA. Plasmid competitor DNAs were prepared by alkaline lysis and sedimentation through cesium chloride gradients (25). pTAd contains the sequence AATTG GCTTGAAGCCAACTAGATC cloned between the *Eco*RI and *Bam*HI sites of pTZ18R (Pharmacia, Inc.). This sequence contains the NFI-binding site from the adenovirus type 5 origin of replication with a point mutation that increases binding about fourfold (36; unpublished results). Plasmids pTZEα and pTZTK contain the oligonucleotides ACTTTTAACCAATCAGAAAAAT and CGAATTCGCCA ATGACAAGACGC, respectively, cloned into the *Sma*I site of pTZ18R. The Eα oligonucleotide contains the CCAAT-binding site for NF-Y/CP1 from the murine major histocompatibility complex class II locus (5), and the TK oligonucleotide contains the CCAAT box from the herpes simplex virus thymidine kinase gene (8).

GMS assay. ^{32}P-labeled oligonucleotide (10 fmol) was incubated with crude nuclear extract (0.3 to 3.6 μg) in 20-μl reaction mixtures containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 5 mM $MgCl_2$, 4 mM dithiothreitol, 150 mM NaCl, 500 μg of bovine serum albumin per ml, 0.05% (vol/vol) Nonidet P-40 (NP-40) (36), 2.5 μg of poly(dI-dC) (Sigma Chemical Co.), 0.005% (wt/vol) bromophenol blue, and 5% (vol/vol) glycerol. The reaction mixtures were incubated at 4°C for 30 min and analyzed on a 0.25× TBE (25)–6.5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide, 30:1). The gels were dried and processed for autoradiography.

Nitrocellulose filter binding assay. The retention of ^{32}P-labeled oligonucleotides (10 fmol) on nitrocellulose filters was measured as previously described (10) under reaction conditions identical to those used in the GMS assay except that bromophenol blue and glycerol were omitted.

UV cross-linking analysis. A 100-fmol amount of ^{32}P-labeled oligonucleotide was incubated with crude nuclear extract (5 to 7 μg) as described for the GMS assay except

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that bromophenol blue and glycerol were omitted. After incubation on ice for 20 min, the samples were UV irradiated (254 nm, 3,000 μW/cm^2, 4°C, 5 min). After irradiation, 10 μl of 3× sodium dodecyl sulfate (SDS) sample buffer mix, (40% [vol/vol] glycerol, 0.19 M Tris chloride [pH 8.8], 9% SDS, 15% [vol/vol] β-mercaptoethanol, 0.01% [wt/vol] bromophenol blue) was added; reaction mixtures were boiled for 5 min and analyzed on a 10% SDS-polyacrylamide gel (22). The gels were dried and processed for autoradiography.

Fractionation of GMS complexes. Binding conditions were similar to those for the standard GMS assay except that 100 fmol of labeled oligonucleotide and 7 μg of HeLa nuclear extract were used. Samples were fractionated as described above on a nondenaturing 6.5% polyacrylamide gel. The gel was UV irradiated (254 nm, 3,000 μW/cm^2, 4°C, 5 min), followed by autoradiography. The shifted bands were excised from the gel, placed in 10 μl of SDS sample buffer mix, boiled for 5 min, inserted into the wells of a 10% SDS-polyacrylamide gel, and subjected to electrophoresis. Samples cross-linked in solution and protein size markers were analyzed alongside the fractionated gel slices.

Cell lines. Nuclear extracts were prepared from the following cultured cell lines: HeLa; CHO (Chinese hamster ovary fibroblast); COS-7 (American Type Culture Collection); NIH 3T3 (American Type Culture Collection); feeder (murine embryonic fibroblast); 9D5-2 (Friend virus-induced erythroleukemia) (28); F-9 (murine embryonic carcinoma) (37); P388D (murine macrophage) (21); 70Z/3 (murine pre-B lymphocyte) (31); SP2-1 (murine myeloma) (43); 2017 (murine immature T cell) (44); and ES (murine D3 embryonic stem cell) (20). In studies of the effects of different growth conditions, NIH 3T3 cells were cultured in α-modified minimal essential medium (GIBCO Laboratories) plus 10% calf serum and 2 mM glutamine. Cells grown to confluence were plated at a high cell density, became confluent within 24 h, and were incubated for an additional 48 h. Cells to be serum starved were plated in medium containing 10% calf serum, incubated for 24 h, washed with serum-free medium, and then incubated in medium containing 0.5% calf serum for 48 h.

Preparation of nuclear extracts. Cells used for the preparation of nuclear extracts were trypsinized and counted. All steps were performed on ice. About 10^7 cells were harvested (1,000 rpm, 5 min; Beckman TH-4 apparatus), washed once in 10 ml of cold phosphate-buffered saline, and suspended in buffer H (25 mM HEPES [pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) at a volume of 0.4 ml of buffer per 10^7 cells. An equal volume of buffer H containing 1% NP-40 was added to lyse the cells, the nuclei were pelleted (2,500 × *g*, 1 min), washed successively as described above with buffer H containing 0.5% NP-40 and with buffer E (25 mM HEPES [pH 7.5], 10% sucrose, 0.01% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), (each at 0.4 ml/10^7 cells), suspended in buffer E containing 0.35 M NaCl (160 μl/10^7 cells), and incubated on ice for 60 min for extraction of nuclear proteins. After extraction, nuclei were removed by centrifugation (10,000 × *g*, 10 min), and the supernatant was decanted and stored at −70°C. Control experiments indicate that these conditions are optimal for the extraction of NFI from nuclei and produce stable extracts free of any inhibitors that might interfere with NFI binding (J. Knox and N. Goyal, unpublished data). For preparation of some extracts, the cells were scraped into cold phosphate-buffered saline rather than trypsinized. Protein concentrations were determined by the method of Bradford (1) (Bio-Rad Laboratories), using bovine serum albumin as a standard.

TABLE 1. Oligonucleotide binding in nuclear extracts[a]

Addition	Oligonucleotide	Amt bound (fmol)
None	FIB-2.6	<0.1
HeLa nuclear extract	FIB-2.6	4.1
	FIB-2.6C2	<0.1

[a] Binding reactions were performed as described in Materials and Methods, using 1 μg of HeLa nuclear extract.

RESULTS

Filter binding assay using nuclear extracts. NFI binds strongly in vitro to the 26-base-pair oligonucleotide FIB-2.6, and a point mutation in the oligonucleotide (FIB-2.6C2) reduces binding about 100-fold (9). We therefore used these two DNAs to quantitate NFI levels in nuclear extracts. The nitrocellulose filter binding assay described previously (9) was modified by including 2.5 μg of poly(dI-dC) in each reaction. This addition greatly reduced nonspecific DNA-binding activity and produced the same difference between the binding of the FIB-2.6 and FIB-2.6C2 DNAs in nuclear extracts (Table 1) as was seen previously with purified NFI (9). The specificity of the assay was tested by competition with various unlabeled plasmid DNAs (Table 2). Control plasmid pTZ18R did not compete for NFI binding, whereas pTAd, which contains the NFI-binding site from the adenovirus type 5 origin of replication, competed effectively for binding to FIB-2.6. In addition, plasmids containing binding sites for the CCAAT-box-binding proteins NF-Y/CPI and C/EBP (2, 5, 8) did not compete for NFI binding. Further control experiments have shown that unlabeled FIB-2.6 DNA competes for the binding seen to FIB-2.6, whereas unlabeled FIB-2.6C2 does not (J. Knox unpublished data).

Different levels of NFI-binding activity in various cell lines. Several different cell lines were grown, and nuclear extracts were made and tested for NFI-binding activity (Table 3). CHO and COS cells had levels of NFI similar to that of HeLa cells, whereas NIH 3T3 cells contained higher amounts of binding activity. Feeder layer cells (upon which ES cells were grown), and Friend cells contained levels of NFI similar to that seen in HeLa cells, whereas P388D, 70Z/3, and SP2-1 cells each contained roughly half as much NFI-binding activity. Interestingly, both ES and F-9 cells,

TABLE 2. Competition for oligonucleotide binding in nuclear extracts[a]

Competitor plasmid	Amt (fmol)	Oligonucleotide bound (%)
None		100[b]
pTZ18R	20	98
	60	93
pTAd (NFI)	20	21
	60	5
pTZEα (NF-Y/CPI)	20	104
	60	110
pTZTK (C/EBP)	20	108
	60	110

[a] Binding reactions were performed as described in Materials and Methods, 1 μg of HeLa extract.

[b] Represents about 5 fmol of FIB-2.6 bound in the absence of competitor DNA.

TABLE 3. Levels of NFI in different cell lines[a]

Cell line	No. of expts	Relative level of NFI (mean ± SD)
CHO	5	1.08 ± 0.13
COS	5	0.73 ± 0.19
NIH 3T3	5	1.49 ± 0.43
Feeder	1	0.89
Friend 9D5-2	5	0.74 ± 0.10
P388D	5	0.47 ± 0.11
70Z/3	1	0.40
SP2-1	6	0.47 ± 0.04
T-cell 2017	5	0.29 ± 0.09
ES	1	0.18
F-9	7	0.11 ± 0.03

[a] Binding assays were performed as described in Materials and Methods in 20-μl reaction mixtures containing 10 fmol of ^{32}P-labeled FIB-2.6 and 0.8 μg of each nuclear extract. The level of NFI in each cell line was divided by the level of binding activity measured in HeLa cell extracts in the same experiment. Binding assays were performed in the linear range of titration curves for each extract, and nonspecific binding to FIB-2.6C2 was <10% of the binding of FIB-2.6 for each extract.

which are undifferentiated embryonal cells, had only about 10% of the level of NFI seen in HeLa cells. Control mixing experiments yielded linear titration curves for the various extracts and showed that none of the extracts contained inhibitors of NFI-binding activity (unpublished data). Nonspecific binding of FIB-2.6C2 was always less than 10% of the specific binding for each extract. These data indicate that cell lines which represent various differentiated tissues possess different total levels of NFI-binding activity.

Multiple NFI-DNA complexes seen in GMS assays. A GMS assay (6) was used to assess the protein complexes formed with the FIB-2.6 and FIB-2.6C2 DNAs in nuclear extracts. Five distinct complexes were formed on the FIB-2.6 DNA by proteins from HeLa nuclear extracts (arrows in Fig. 1, lane 2). No complexes were seen in the absence of extract and when FIB-2.6C2 was used as a probe (lanes 1 and 3, respectively). As with the filter binding assay, binding was not abolished by a 1- or 10-fold molar excess of pTZ18R (lanes 4 and 5), whereas 1- and 10-fold molar excesses of pTAd competed very efficiently (lanes 6 and 7).

Multiple cross-linked NFI polypeptides. To analyze the apparent size distribution of the polypeptides that interact with FIB-2.6 DNA, a UV cross-linking assay was developed (15, 33). This technique creates covalent links between DNA and the amino acid side chains of a binding protein (3, 16, 26, 40). Denaturing of the samples and fractionating on a SDS-polyacrylamide gel eliminated noncovalent protein-protein interactions and measured the approximate size and number of NFI species interacting directly with the DNA (Fig. 2). No cross-linking was seen in the absence of either extract or UV irradiation (Fig. 2, lanes 1 and 2). However, when FIB-2.6 was irradiated in the presence of HeLa nuclear extract, a number of bands with apparent sizes of 38 to 70 kDa were detected, with a major band at around 68 kDa (lane 3; arrows depict most clearly distinguished bands). Repeated analyses showed six to eight distinct FIB-2.6–protein covalent complexes to be present after UV cross-linking. No bands were seen in corresponding regions of lanes containing the mutant oligonucleotide FIB-2.6C2 (lane 4). The species with higher molecular size and the small band of around 28 kDa that bound to both DNAs were apparently due to low levels of nonspecific DNA-binding activity in the extracts. The specificity of the assay was further demonstrated by showing that FIB-2.6 and pTAd, but not FIB-

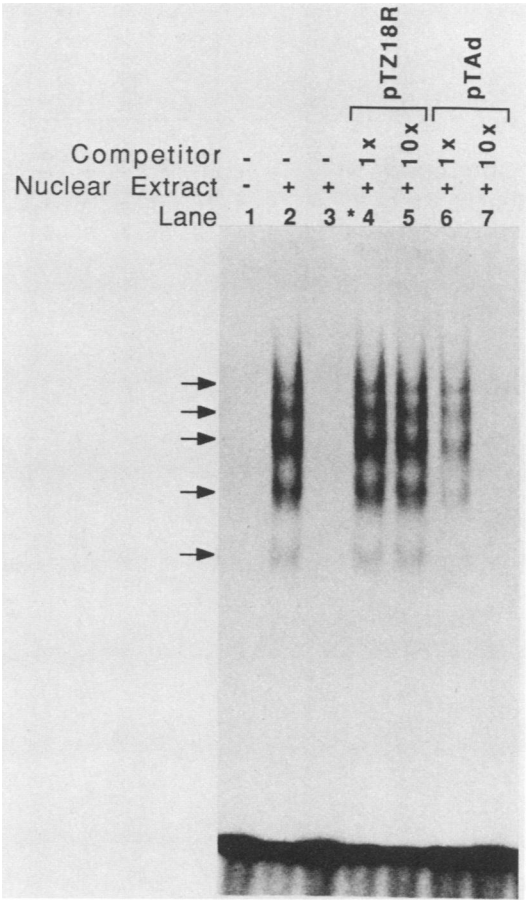

FIG. 1. Detection of NFI in nuclear extracts by GMS analysis. A GMS assay was performed as described in Materials and Methods in 20-μl reaction mixtures containing nuclear extraction buffer (lane 1) or 1 μg of HeLa nuclear extract (lanes 2 to 7). All reactions contained 10 fmol of ^{32}P-labeled FIB-2.6 oligonucleotide except lane 3, which contained 10 fmol of ^{32}P-labeled FIB-2.6C2. Lanes contained competitor DNAs as follows: 4 and 5, 1- and 10-fold molar excess, respectively, of pTZ18R; 6 and 7, 1- and 10-fold molar excess, respectively, of pTAd. Major complexes detected are indicated by arrows. Free oligonucleotide is seen near the bottom of the gel.

2.6C2, were able to compete efficiently for NFI cross-linking (lanes 5 to 9). Numerous independent extract preparations have yielded consistent cross-linking results. Presumably because of the small size of the oligonucleotide used here, DNase I treatment before denaturation had no effect on the apparent sizes of the covalent protein-DNA complexes seen (unpublished results). Various control experiments indicated that the extracts were extremely stable, showing no changes in the forms of cross-linked protein detected upon multiple freeze-thawing or preincubation of the extracts at 37°C for 30 min (data not shown).

Unequal distribution of NFI species in GMS complexes. To determine the composition of NFI polypeptides in each GMS complex, the complexes were fractionated on a nondenaturing polyacrylamide gel (6, 39, 47), the gel was UV irradiated, and the GMS complexes were excised, boiled in SDS buffer, and analyzed by SDS-polyacrylamide gel electrophoresis. Since the frequency of cross-linking is very low (<5% [15, 33]), the probability of having more than one NFI monomer cross-linked to the same DNA fragment is negli-

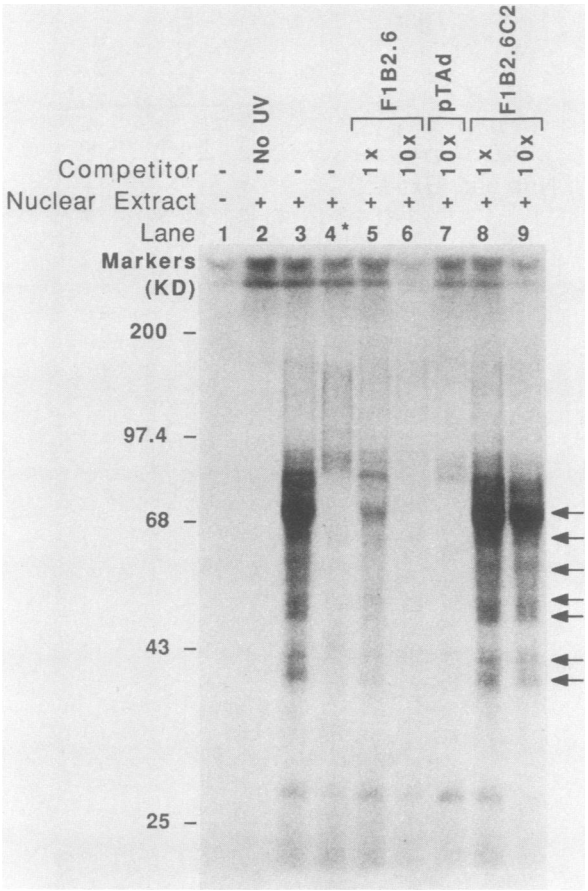

FIG. 2. Detection of NFI polypeptides by a UV cross-linking assay. HeLa nuclear extract (4 μg of protein) was analyzed by UV cross-linking as described in Materials and Methods in 20-μl reaction mixtures. All lanes contained 100 fmol of ^{32}P-labeled FIB-2.6 except lane 4, which had 100 fmol of ^{32}P-labeled FIB-2.6C2. Lanes: 1, minus nuclear extract; 2, minus UV irradiation; 3 to 9, 4 μg of nuclear extract and irradiation. Lanes contained competitor DNAs as follows: 5 and 6, 1- and 10-fold molar excess, respectively, of unlabeled FIB-2.6; 7, 10-fold molar excess of pTAd; 8 and 9, 1- and 10-fold molar excess, respectively, of unlabeled FIB-2.6C2. The most readily detected bands are indicated by arrows.

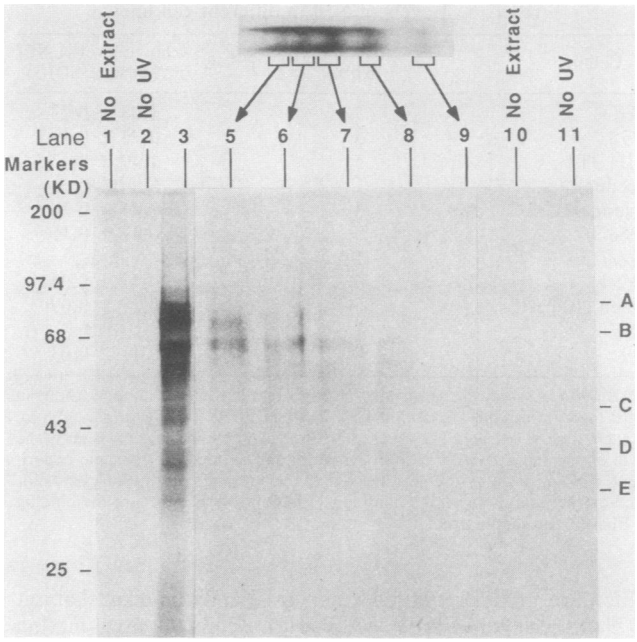

FIG. 3. Fractionation of GMS complexes. GMS complexes were fractionated on a nondenaturing polyacrylamide gel, UV irradiated, and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. All reaction mixtures contained 100 fmol of ^{32}P-labeled FIB-2.6 DNA. Lanes 1 to 3 show samples cross-linked in solution; lanes 4 to 11 show complexes excised from the nondenaturing gel. Lanes 1 and 2 were prepared as indicated. Lanes 5 to 9 each show one of the five excised GMS complexes, from the slowest to the fastest migrating, respectively (shown from left to right in the horizontal lane above the main gel). The cross-linked peptides most readily detected are labeled A through E for illustration. Lanes 10 and 11 show fractionation experiments done in the absence of nuclear extract and UV irradiation respectively. KD, Kilodaltons.

gible in this assay. In the absence of nuclear extract or UV irradiation, no bands were detected (Fig. 3, lanes 1, 2, 10, and 11). The most slowly migrating GMS complex was composed predominantly of two equal-intensity large polypeptides, bands A and B, cross-linked to the probe (lane 5). Proceeding from the slowest- to the fastest-migrating GMS complex, there was a decrease in the abundance of the larger-molecular-weight forms. The presence of band B in a majority of the complexes may reflect the binding of a common monomer of NFI to one of the two motifs. Other bands were seen only in particular complexes (i.e., band E seen only in lane 6) and may represent distinct subforms of NFI binding to the second of the two motifs.

Different NFI species in various murine cells. Since different amounts of NFI were seen in various cell lines (Table 3), we examined the forms of NFI present in each cell type by using the GMS assay (Fig. 4). Regions of the gel containing the major complexes detected were labeled A through H for illustration and discussion purposes. ES and F-9 extracts contained several NFI-DNA complexes (Fig. 4, lanes 2 and 5). Some complexes were common to both cell lines (regions B and C), whereas others were unique to each line (regions E and G in ES cells; regions D and F in F-9 cells). The feeder cell layer on which the ES cells were grown contained complexes in regions E, G (common to ES cells), and H (unique complex). 70Z/3 cells contained complexes predominantly in region B, with trace amounts at A (lane 11). SP2-1 cells also contained a predominant complex in region B, with low levels of other complexes in regions A, C, and D (lane 14). P388D cells possessed a major complex in region C, with minor forms in regions B and D (lane 17). Finally, the T-cell line contained complexes only in regions A and B (lane 20). These last four cell lines are all at least partially differentiated and possess a limited number of complexes relative to the less differentiated lines ES and F-9.

Growth state of cells has an effect on forms of NFI seen. We next investigated the effect of cellular growth state on the forms of NFI present. NIH 3T3 cells were harvested during exponential growth, after 48 h of serum starvation, and after growth to confluence. Extracts were made and analyzed by a GMS assay (Fig. 5). Cells growing exponentially contained three major complexes (Fig. 5, lane 2; regions C, D, and E). Upon growth to confluence, there was a shift to slower-migrating species (predominantly in region B, with trace amounts at A; lane 3), along with a decrease in the abundance of complexes in regions C and D. Serum starvation of the cells resulted in a slight increase in the levels of complexes in regions B and C relative to the control (lane 4).

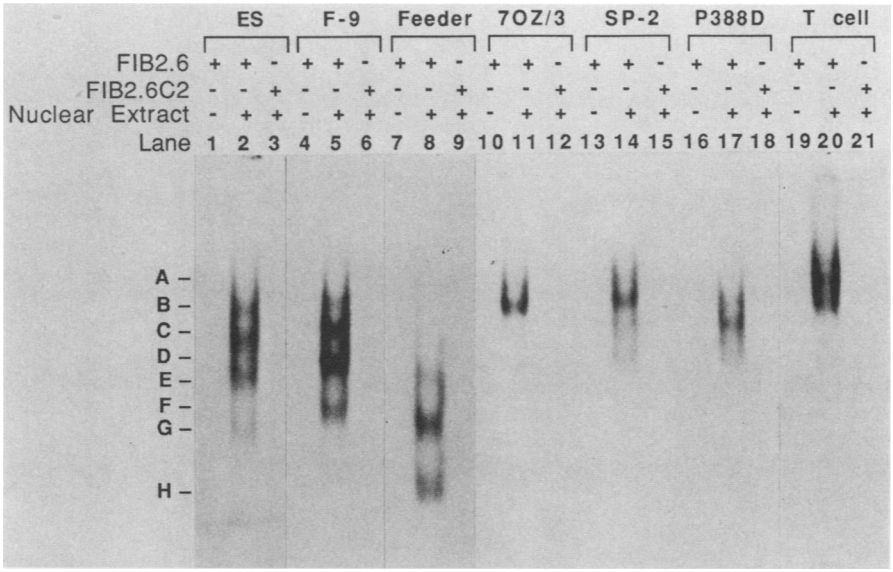

FIG. 4. GMS analysis of different murine cell lines. GMS assays were performed with nuclear extracts from several murine cell lines. Lanes: 1, 4, 7, 10, 13, 16, and 19, minus nuclear extract plus labeled FIB-2.6 DNA; 2, 5, 8, 11, 14, 17, and 20, plus nuclear extract plus labeled FIB-2.6 DNA; 3, 6, 9, 12, 15, 18, and 21, plus nuclear extract plus labeled FIB-2.6C2 DNA. Lanes contained nuclear extract as follows: 2 and 3, 2 μg of ES; 5 and 6, 3.6 μg of F-9; 8 and 9, 0.45 μg of feeder; 11 and 12, 0.96 μg of 70Z/3; 14 and 15, 0.8 μg of SP2-1; 17 and 18, 0.8 μg of P388D; 20 and 21, 1.6 μg of T cell (cell line 2017). The T-cell lanes were exposed three times longer than the ES, F-9, and feeder lanes, and the 70Z/3, SP2-1, and P388D lanes were exposed for half as long. Major complexes seen are labeled A through H for illustration only.

Growth arrest was verified by cell cycle analysis using DNA cytofluorimetry (N. Goyal, unpublished results).

To determine whether the difference seen in the GMS complexes during growth arrest was accompanied by a comparable shift in the apparent size of NFI polypeptides interacting with DNA, nuclear extracts were assayed by UV cross-linking (Fig. 6). No cross-linking was seen in the absence of extract or UV irradiation (Fig. 6, lanes 1, 4, 7, and 10), whereas after irradiation a number of peptide species (with apparent sizes of between 35 and 70 kDa) were cross-linked to FIB-2.6 (lanes 2, 5, and 8) but not to FIB-2.6C2 (lanes 3, 6, and 9). Extracts from confluent cells showed an increased abundance in slower-mobility cross-linked polypeptides that correlated with the observed shift to slower-migrating GMS complexes (Fig. 6, lanes 2 and 5, compared with Fig. 5, lanes 2 and 3). Extracts made from serum-starved cells showed only a minor increase in larger cross-linked forms of NFI, which also was detected on GMS assays (Fig. 6, lanes 2 and 8, versus Fig. 5, lanes 2 and 4). The smaller-size bands (at 23 and 25 kDa) that cross-linked to both probes was apparently due to low levels of nonspecific DNA-binding activities (lanes 2, 3, 5, 6, 8, and 9). These data indicate that the forms of NFI present in NIH 3T3 cells are altered by both the growth state of the cell and the method used to arrest cell growth.

DISCUSSION

These and other studies (7, 19, 27, 32, 35, 41) demonstrate that NFI is made up of a family of proteins that bind to specific sites on DNA. We have shown that both the forms (Fig. 4) and absolute levels (Table 3) of NFI vary in different cell lines.

Previous studies have shown that NFI purified from HeLa cells consists of a group of proteins ranging in molecular size from 55 to 62 kDa (19, 35). However, it is possible that some of the polypeptides observed were generated by proteolysis during purification. To reduce the likelihood of proteolytic degradation, we have used nuclear extracts in our binding assays. NFI appears to be quite stable in extracts prepared as described in Materials and Methods. Neither multiple freeze-thawing nor incubation of the extracts at 37°C for 30 min had any effect on the apparent forms of NFI observed by GMS or UV cross-linking assays (N. Goyal, unpublished results). No appreciable differences were seen in repeated independent extract preparations. In addition, numerous control mixing experiments of both different nuclear extracts and different cell lines indicated that there was no interconversion of the forms of NFI during extract preparation (N. Goyal, unpublished results). As discussed earlier, these extracts also appear free of any proteolytic activity or inhibitors that might interfere with GMS or UV cross-linking assays.

Specificity of binding. All of our studies used a small oligonucleotide (FIB-2.6) previously shown to interact strongly and specifically with NFI (10). A point mutation in the first motif (TGG→TCG) abolishes binding, as judged by retention on nitrocellulose filters (Table 1), GMS analysis (Fig. 1), and UV cross-linking (Fig. 2). The second motif of the binding site, which contains a CCAAT box, by itself is apparently not recognized by other factors in the nuclear extracts (Tables 1 and 2; Fig. 1, 2, and 4 to 6). These findings are consistent with previous studies on other CCAAT-box-binding proteins (NF-Y/CP1, CP2, and C/EBP), which showed that each of these proteins binds to distinctive recognition sequences (2, 5, 8).

The minimum sequence requirement for weak binding of NFI is uncertain. Some studies have detected NFI binding to what can be described as half sites, i.e., single motifs of TGG or GCCAA (7, 19, 32). In the studies described here, a point mutation in the TGG motif of FIB-2.6 effectively abolishes binding (Table 1; Fig. 1 and 2). Such apparent differences in the sequence requirements for NFI binding are most likely

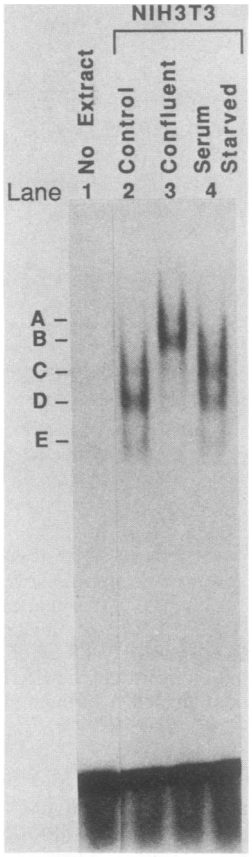

FIG. 5. Effect of growth condition on NFI GMS complexes. Nuclear extracts (0.6 μg) from NIH 3T3 cells that were exponentially growing (lane 2), grown to confluence (lane 3), or serum starved (lane 4) were analyzed by GMS assay. All lanes contained 10 fmol of ^{32}P-labeled FIB-2.6 DNA. Free DNA is seen at the bottom of the gel. Major complexes are labeled A through E for illustration only.

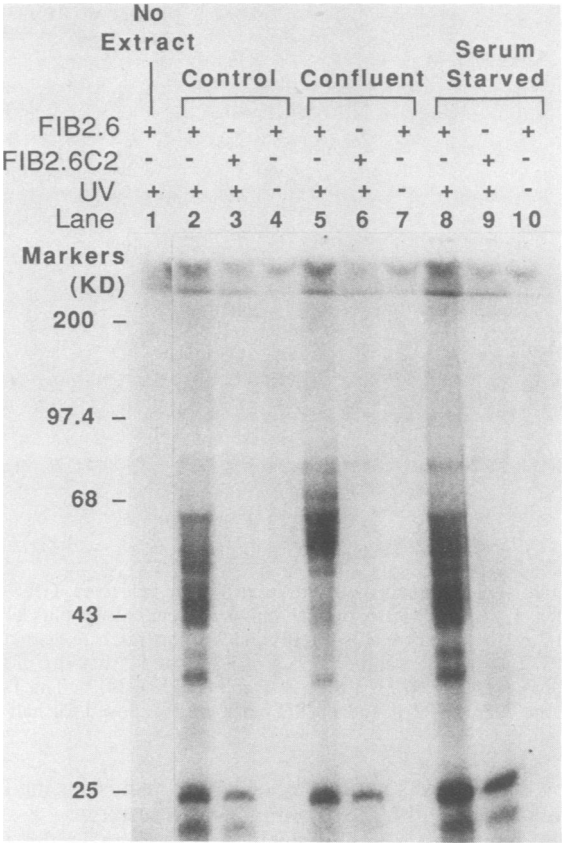

FIG. 6. Effect of growth condition on NFI UV cross-linking products. NIH 3T3 extracts from Fig. 5 were assayed by UV cross-linking. Lanes: 1, minus nuclear extract; 2 to 4, 3.5 μg of control cell extract; 5 to 7, 3.5 μg of confluent cell extract; 8 to 10, 3.5 μg of serum-starved cell extract. Reactions contained 100 fmol of ^{32}P-labeled FIB-2.6 DNA except for lanes 3, 6, and 9, which contained ^{32}P-labeled FIB-2.6C2 DNA. Lanes 4, 7, and 10 were not UV irradiated.

due to differences in the conditions of the assays used to measure binding.

Multiple GMS complexes. In HeLa nuclear extracts, we have resolved five distinct NFI-DNA GMS complexes (Fig. 1). There are at least three ways that such multiple complexes might arise. As discussed earlier, purified NFI consists of a heterogeneous group of proteins, and multiple mRNA species encoding NFI have been identified in HeLa cells and other cell types (27, 32, 41). In addition, several studies indicate that NFI binds to DNA as a dimer (4, 7, 11, 27). Therefore, the combination of different-size subunits of NFI (as either homodimers or heterodimers) binding to a single site could generate multiple GMS complexes. A second possibility is that proteins are present in the nuclear extracts which bind to the NFI-DNA complex via protein-protein interactions with NFI. For example, the adenovirus E1a protein has no apparent DNA-binding ability but appears to regulate transcription by interacting with other proteins already bound to DNA (24). Such a protein might interact with NFI bound to its recognition site. Finally, a combination of the above two mechanisms may produce multiple GMS complexes.

Multiple cross-linked NFI species. To detect only proteins in direct contact with the NFI-binding site, a UV cross-linking assay was developed (Fig. 2, 3, and 6). UV light is a zero-length cross-linker that freezes contact points in protein-DNA interactions (16). By using 254-nm light, only protein-DNA cross-links occur (3, 26, 40). We have shown that multiple polypeptides from HeLa nuclear extracts cross-link to FIB-2.6 DNA (Fig. 2). Only very low levels of cross-linking were detected to the mutant oligonucleotide (FIB-2.6C2). This low level of cross-linking is likely due to nonspecific DNA-binding proteins that interact with the mutant site. Nonspecific DNA-binding proteins can cross-link to a variety of DNAs (15, 26, 33). Presumably, these nonspecific protein-DNA interactions are relatively weak and not detected by a GMS assay (Fig. 1) but can be trapped by UV cross-linking (Fig. 2).

We have shown that the different GMS complexes contain different-size polypeptides that cross-link to FIB-2.6 DNA (Fig. 3). Also, a single GMS complex can contain multiple forms of NFI (lane 6). Furthermore, some complexes with different mobilities in a GMS assay appear to contain similar NFI species cross-linking to DNA (lanes 7 and 8). Further studies are needed to determine whether the structural heterogeneity of NFI-DNA complexes is indicative of functional heterogeneity of the complexes.

Cell type specificity in amounts and forms of NFI. Differences in both the amounts and forms of NFI were seen across different species (Table 3) and between cell lines of

the same species (Table 3 and Fig. 4). However, in comparisons between cell lines, GMS complexes with similar mobilities may not have identical compositions. As discussed above, combinations of different-size monomers of NFI, additional protein-protein interactions, or both may generate complexes with the same apparent mobility but different polypeptide compositions. The mechanism responsible for the generation of different forms of NFI in different cell types is not known; however, a number of possibilities exist. (i) Multiple RNA species encoding NFI have been detected in several species (7, 27, 32, 41). It is unclear at present whether all of these mRNAs are translated into functional proteins. (ii) NFI polypeptides may be subject to differential posttranslational modifications, such as the O-linked glycosylation seen in HeLa cells (17). (iii) Changes in the stability of the RNA and protein species may occur in different cell lines, which could alter the levels of different forms of NFI.

Growth condition-induced changes in NFI. Our analysis of NIH 3T3 cells indicates that the forms of NFI vary with the growth condition of the cells (Fig. 5 and 6). However, extracts prepared under all growth conditions contained about the same level of overall binding activity. A question of major interest is: What is the role of the various species of NFI detected? Since we are measuring only the DNA-binding ability of NFI, the functions of these various forms of NFI in RNA synthesis are not known. It seems likely that the functions of the distinct forms of NFI that we have detected may differ. For example, some forms of NFI may act as repressor molecules, whereas others may activate gene expression. It is possible that the same form of NFI could perform both functions and selectively activate or repress the expression of different genes. In addition, the alignment of multiple binding sites for different site-specific DNA-binding proteins in various promoters may pose a steric limitation on which form(s) of NFI could bind at a particular promoter. Thus, different subsets of genes may bind only specific forms of NFI. Differential gene expression could then occur through changes in the forms of NFI present in the cell.

Some forms of NFI are O glycosylated (17). The existence of such modifications suggests that the function of NFI may be regulated by posttranslational mechanisms. Since we are measuring only the forms of NFI that can bind to DNA, any modification of NFI that disrupts its DNA-binding ability would not be detected in these assays. Such non-DNA-binding forms of NFI could also disrupt the function of the DNA-binding forms of NFI by creating nonfunctional heterodimers of the protein analogous to *lacI*$^{-d}$ mutations in the *lac* repressor (45). Clearly, it will be important to determine whether such modifications of NFI play a role in its regulation.

The final potentially important finding of this study is that the method used to arrest cell growth affects the forms of NFI present (Fig. 5 and 6). It appears that regulation of the forms of NFI present within cells is acutely sensitive to changes in the cellular environment. The significance of this observation requires further investigation. In addition, further studies are needed to assess the function of the various forms of NFI that we have detected and to determine the mechanism(s) by which each form is generated.

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