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Nuclear Factor 1 Family Proteins Bind to the Silencer Element in the Rat Glutathione Transferase P Gene[1]

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We have previously identified a silencer region in the glutathione transferase P (GST-P) gene, of which the expression is completely repressed in liver of the rat. At least three *trans*-acting factors bind to multiple *cis*-elements in this region. Since GST-P silencer 4 (GPS4) is a dominant element in this silencer, we purified the GPS4 binding protein, called Silencer Factor A (SF-A). Purified SF-A was separated into several proteins on an SDS-polyacrylamide gel, and the amino acid sequences of four major components of SF-A were determined. The amino acid sequences of three fragments were identical to those of rat NF1-L, and that of the other fragment was the same as that of hamster NF1/Red1. It is known that nuclear factor 1 (NF1) family proteins are encoded by at least four independent genes in vertebrates, and NF1-L and NF1/Red1 are derived from different genes, NFI-A and NFI-B, respectively. The microsequencing of SF-A revealed that at least two types of NF1 existed in rat liver. Functional analysis by using GAL4-fusion protein in HepG2 cells revealed that NFI-A repressed the transcription activitiy from human metallothionein II_A promoter. Our findings indicate that multiple forms of the NF1 family bind to the silencer region and contribute to the negative regulation of the GST-P gene expression.

Key words: GST-P, nuclear factor 1, purification, silencer element, transcription factor.

The rat glutathione transferase P (GST-P) is strongly and specifically expressed during chemical hepatocarcinogenesis and is considered to be an excellent tumor marker (*1*). This gene consists of an enhancer and a silencer. The strong enhancer located at 2.5 kb upstream from the cap site plays a crucial role in the tumor-specific expression of this gene (*2-6*). The negative regulatory element located at 400 bp upstream from the cap site is also significant, and our understanding of this element would help clarify the repression mechanism of the GST-P gene in normal rat liver (*2, 7*).

Footprint analysis using rat liver nuclear extracts and transfection analysis using cultured cells demonstrated that multiple *cis*-elements and multiple *trans*-acting factors are involved in the negative function in the silencer of the GST-P gene. *In vivo* competition and internal deletion analyses of each *cis*-element resulted in a decrease in the promoter activity (*7*). Therefore, each element and the binding factors play important roles in the silencing activity. One of the binding factors, Silencer Factor B (SF-B), which binds to GST-P silencer 1 (GPS1), was identified previously as a member of the CCAAT/enhancer binding protein (C/EBP) family (*8*). The ratio of C/EBPα to C/EBPβ is one of the important factors for the GST-P silencer activity, and the decrease of this ratio during hepatocarcinogenesis reduces the silencer activity, and consequently increases the GST-P expression (*9*). The repression activity of SF-B might be influenced by Silencer Factor A (SF-A) and Silencer Factor C (SF-C), which bind to the silencer elements near the GPS1 site. Since the previous studies indicated that GST-P silencer 4 (GPS4) seemed to be the most functional element in this region (*7*), we purified the GPS4 binding protein, called SF-A, and found that partially purified SF-A bound to several regions in this silencer (*7*). A schematic diagram of SF-A binding sites in the promoter and silencer regions in the GST-P gene is shown in Fig. 1.

In the present study, we demonstrate the purification and the partial amino acid sequences of SF-A. All sequences of the determined peptides were identical to NF1-L/NFI-A (*10*), except for one sequence which was the same as hamster NF1/Red1 (*11*); both are members of the nuclear factor 1 (NF1) transcription factor family. In addition, the functional analysis using GAL4-fusion protein revealed that NFI-A repressed the transcription in HepG2 cells, indicating that NFI-A is a negative regulator.

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; GPS1, GST-P silencer 1; GPS4, GST-P silencer 4; GST-P, glutathione transferase P; MT, metallothionein; NF1, nuclear factor 1; PCR, polymerase chain reaction; SF-A, Silencer Factor A; SF-B, Silencer Factor B; SF-C, Silencer Factor C.

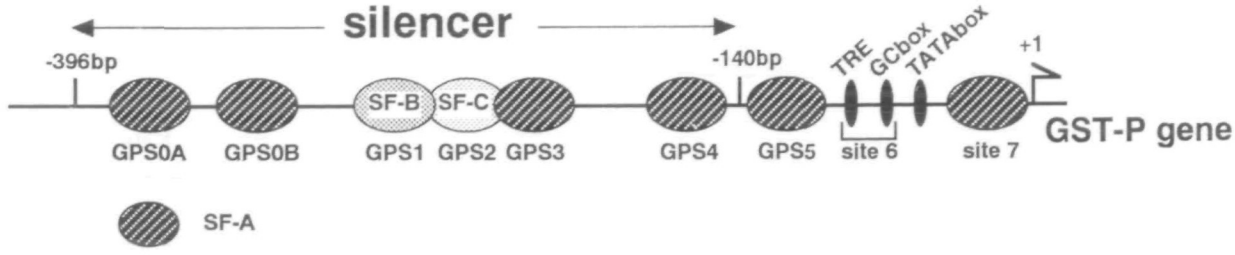


Fig. 1. **A schematic diagram of SF-A binding sites in the promoter and silencer regions in the GST-P gene.** SF-A binding sites as well as SF-B and SF-C are shown. The silencer region was originally identified as a *Bam*HI-*Pst*I fragment located from −396 to −140 (*7*).

MATERIALS AND METHODS

Purification and Microsequencing of SF-A—Rat liver nuclear extracts were prepared according to the method of Lichtsteiner *et al.* (*12*) as described previously (*7*). For SF-A purification, the buffer used in later steps was Buffer Z, consisting of 25 mM HEPES (pH 7.9), 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 20% glycerol, 0.1% Nonidet P-40, and KCl as indicated. Nuclear extracts obtained were loaded on a column of Ultrogel AcA44 equilibrated with 0.1 M Buffer Z, and separated fractions were assayed for GPS4 DNA-binding activity by gel shift assay. SF-A fractions were heated at 55°C for 5 min, then centrifuged. The supernatant was loaded onto a Heparin-Sepharose CL-6B column (Pharmacia Biotech) and eluted with 0.2, 0.4, and 0.6 M Buffer Z. The active fractions were pooled and dialyzed overnight against 0.1 M Buffer Z, then poly (dI-dC) was added to a final concentration of 50 μg/ml. After incubation on ice for 10 min, the mixture was loaded onto a GPS4-affinity column (*7*). The column was washed with 0.1 M Buffer Z and eluted with 0.6 M Buffer Z. This step was repeated three times, then SF-A fractions were precipitated with five volumes of acetone at −20°C and centrifuged. Purified SF-A was separated on SDS-polyacrylamide gel, and the four major proteins were digested with lysyl endopeptidase (Wako Pure Chemicals, Osaka) at 37°C for 6 h. The resulting peptides were separated by reversed phase HPLC and sequenced. Amino acid sequences from several peptides in each protein were determined.

Western Blot Analysis—Purified SF-A was separated on SDS-polyacrylamide gel and detected by anti-NFI-A antibody (*13*), kindly provided by Dr. P.C. van der Vliet. Western blot analysis was performed as described previously (*9*).

Library Screening and DNA Sequencing—^{32}P-labeled mouse NFI-B1 cDNA (*14*) kindly provided by Dr. T. Tamura (Chiba University) was initially used as a probe to screen 3×10^6 phages of rat liver λgt11 cDNA library (Clontech), and only one clone (λ4) was isolated. To obtain the full-length cDNA clones, the insert of λ4 was used as a probe for further screening of 3×10^6 phages of the rat liver λgt10 cDNA library kindly provided by Dr. N. Miura (Akita University). We obtained 32 independent clones, and each insert of all isolated clones was subcloned into a pBluescript vector. The nucleotide sequence was determined by an automated DNA sequencer DSQ 1000 (Shimadzu, Kyoto) and the dideoxy method by using ^{32}P (*15*).

Reverse Transcriptase-Coupled Polymerase Chain Reaction (PCR) Analysis—Total RNA was isolated from rat liver using ISOGEN (Nippon Gene, Tokyo) and treated with deoxyribonuclease I. The reverse transcriptase reaction was carried out for 30 min at 55°C using 1.5 μg of total RNA as a template. The following primers were used for PCR analysis: S-88/S-95, position 394-696 bp; S-88/S-99, position 394-1068 bp; S-66/S-64, position 1321-1553 bp (Fig. 4). The PCR amplification was performed over 30 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. The reaction products were separated on a 6% polyacrylamide gel and stained with ethidium bromide. The positive fragments were recovered, blunted, cloned into the *Sma*I site of the pBluescript vector, and sequenced by the dideoxy method (*15*).

Plasmid Constructs—For the expression of NFI-A as a histidine fusion protein (QIAGEN), a *Bam*HI-*Sph*I fragment derived from λ4 in pBluescript encoding 5-318 amino acids was subcloned into a pQE-30 expression vector.

A PCR-based method was used to create GAL4-NFI-A1, -A2, -A3, and -A4. *Eco*RI sites at the translation initiation site and *Sal*I sites at the stop codon of NFI-A1, -A2, and -A3 cDNAs were created by PCR. *Eco*RI-*Sal*I fragments containing the open reading frames of NFI-A1, -A2, and -A3 were subcloned into the *Eco*RI-*Sal*I site of the pBluescript vector. The stop codon of NFI-A4 was also created by PCR with a primer corresponding to the sequence from 1607 to 1583. NFI-A4 in pBluescript was obtained by inserting a *Sph*I blunt-end fragment from NFI-A4 into NFI-A1 in pBluescript digested with *Sph*I-*Hin*cII. *Sca*I-*Hin*cII fragments from NFI-A1, -A2, and -A3, or *Sca*I-*Xho*I filled in a fragment from NFI-A4 were subcloned into pSG424, a GAL4 DNA-binding domain expression plasmid, at the *Sal*I-fill-in site in the vector (*16*). All fragments generated by PCR were checked by sequencing by the dideoxy method (*15*).

For the construction of 5xGAL4-$MTII_A$-luciferase, a GAL4 binding site was synthesized and multimerized to a 5-mer, then cloned into the *Xho*I site of pBluescript KS^+. Metallothionein ($MTII_A$) promoter harboring −764 to +69 (*17*) was inserted into the *Hin*dIII site in the luciferase vector, PGV-B (Toyo Ink). Then, the *Kpn*I-*Bam*HI fragment including the 5-mer of the GAL4 binding site was cloned into the *Kpn*I-*Bgl*II site of the $MTII_A$-luciferase construct. The sequence of the GAL4 binding site of the upper strand was as follows: 5′-TCGACGGAAGACTCTC-CTCCGT-3′.

Expression and Purification of Recombinant NFI-A—The NFI-A DNA-binding domain expression plasmid was transformed into M15 [pREP4]. The transformant was grown overnight at 30°C in a Luria-Bertani (LB) agar plate containing 100 μg/ml ampicillin and 25 μg/ml kanamycin,

inoculated with 100 ml of LB medium from the plate, and grown to OD_{600} 0.8; at that time isopropyl-β-D-thio-galactopyranoside was added to a final concentration of 0.1 mM. The cells were then allowed to grow for an additional 4 h at 25°C, harvested by centrifugation and suspended in 10% of the original culture volume in 0.5 M B buffer consisting of 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% glycerol, 0.1% Nonidet P-40, 1 μM pepstatin, 4 μM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and KCl as indicated, and disrupted by sonication. Following centrifugation at 98,000 rpm for 1 h, the supernatant was mixed with Ni^{2+}-nitrilotriacetic acid-agarose and rotated at 4°C for 15 min. NFI-A was eluted with 0.5 M B buffer containing 0.25 M imidazole. The eluate was dialyzed overnight against 0.1 M Buffer Z.

DNase I Footprint Analysis and Gel Shift Assay—DNase I footprint analysis was performed as described (7). The sequences of oligonucleotides for gel shift assay were as follows (only upper strands are shown):

NF1-adeno: 5′-CTAGCTATTTTGGATTGAAGCCAATATG-3′
GPS4: 5′-CTAGTTTCTTGGAGCAGGACCCAAAAAT-3′
GPS4: 5′-CTAGAGAGGTTGGTAAATAGGGATGG-3′

The protein fraction (6.25 μl) containing the 15 μg of rat liver nuclear extracts was mixed with the same volume of 20 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, 10 mM EDTA, 0.2 ng labeled probe, 2 μg poly(dI-dC), and 0.1% Nonidet P-40. When NFI-A antibody was used for super-shift analysis, the nuclear extracts were pre-incubated for 30 min with the antibody prior to the addition of other reagents. Each reaction mixture was loaded on 6% non-denaturing polyacrylamide gel, electrophoresed at 150 V for 1 h, fixed with 10% methanol and 10% acetic acid, and autoradiographed at −80°C.

Cell Culture, Transfection, and Luciferase Assay—HepG2 cells, a human hepatoma cell line, were cultured in minimal essential medium supplemented with 10% fetal bovine serum, and passaged by trypsinization at confluence. Transfection was carried out by the calcium phosphate co-precipitation technique (*18*), using 3.5 μg of the luciferase reporter plasmid, 1.0 μg of effector plasmid, and 0.5 μg of pRSVGAL, a eukaryotic expression vector that contains the *Escherichia coli* β-galactosidase (lacZ) structural gene controlled by the Rous sarcoma virus long terminal repeat, as an internal control. Luciferase activities were measured by use of Pikka Gene (Toyo Ink). All the transfection experiments were performed at 13 to 16 times by using two different preparations of DNA, and the relative luciferase activity was derived from the mean values of the results. The activity of β-galactosidase was assayed as described (*19*).

RESULTS

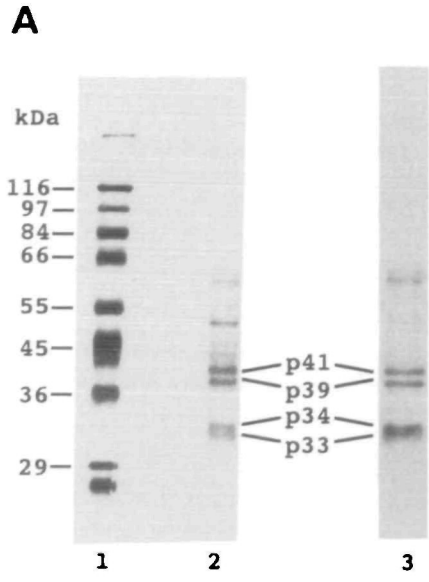
Purification of SF-A from Rat Liver Nuclear Extracts—Rat liver nuclear extracts contain an SF-A activity capable of sequence-specific interaction with GPS4 in the GST-P silencer. Using a combination of chromatographic techniques coupled with gel shift assay by using GPS4 as a probe, SF-A was purified. Table I shows quantitative estimates of the purification at each step, starting with nuclear extracts from 12 rats. One unit of activity was defined as the activity that shifts 10% of the specific probe under the standard assay conditions. Crude nuclear extracts of the rat liver were applied first to an Ultrogel AcA44. Approximately 60% of the total protein was recovered in the void volume of the column. Since the Ultrogel AcA44 fractions contained some exo- and endonuclease activities endogenously and SF-A activity was heat-stable (7), the fractions containing SF-A activity were heated at 55°C for 5 min and then centrifuged, and the supernatant was loaded onto a Heparin-Sepharose column and eluted at 0.4 M KCl. To complete the purification of the SF-A, we performed the GPS4-affinity chromatography. Since the eluate of the first GPS4-affinity column contained many kinds of protein, the eluate was again applied to an affinity column. After three passages through the GPS4-affinity column, the eluate was precipitated with acetone, subjected to SDS-polyacrylamide gel electrophoresis, and Coomassie Brilliant Blue staining. A single band of SF-A was not obtained on SDS-polyacrylamide gel electrophoresis; rather, several proteins including four major components (p41, p39, p34, and p33) were detected (Fig. 2A).

Partial Amino Acid Sequences of SF-A (p41, p39, p34, and p33)—SF-A was purified as several proteins from rat liver nuclear extracts. The four major components, p41, p39, p34, and p33, were separated on SDS-polyacrylamide gel and digested with lysyl endopeptidase. The peptides were separated by reversed phase HPLC and sequenced. The peptide elution profiles of p41, p39, and p34 on reversed phase HPLC were similar (data not shown). Amino acid sequences from several peptides in each protein were determined (Fig. 2B). The amino acid sequences from p41, p39, and p34 were identical to the predicted sequence from rat NF1-L cDNA, and the sequence from p33 coincided with the predicted sequence from hamster NF1/Red1 cDNA. NF1-L and NF1/Red1, members of the NF1 family, were purified from rat and hamster liver nuclear extracts, respectively (*10, 11*). NF1 proteins in vertebrates are encoded by a set of four genes (NFI-A, NFI-B, NFI-C, and NFI-X), which have been highly conserved (*11, 20*). To avoid confusion, rat NF1-L (*10*) including p41, p39, and p34 derived from rat NFI-A gene is named NFI-A, whereas p33 is called NFI-B, because NF1/Red1 is encoded by the NFI-B gene. To confirm that the purified SF-A fraction contains NF1 proteins, we next performed Western blot analysis using by the anti-NFI-A antibody (Fig. 2A, lane 3).

TABLE I. **Purification of SF-A from rat liver.**

Step	Protein (mg)	Total activity[a] (units)	Specific activity[a] (units/mg)	Purification (-fold)	Recovery (%)
Nuclear extract	210	28,560	136	1	100
Ultrogel AcA44	70	22,050	315	2.32	77.2
Heat (55°C, 5 min)	39	15,720	401	2.95	55.0
Heparin-Sepharose	7.5	5,617	753	5.54	19.7
GPS4-affinity (1st)	0.11	3,575	32,500	240	12.5
GPS4-affinity (2nd)	0.04	3,312	82,800	609	11.6
GPS4-affinity (3rd)	0.02	2,860	143,000	1,051	10.0

[a]One unit is defined as the activity causing the shift of 10% of the labeled GPS4 under the standard assay conditions.



B

Protein	Amino acid sequence	Position
p41	YFK	36-38
(NF1-L)	EEERAVKDELLSEKPEVK	47-64
p39	YFK	36-38
(NF1-L)	EEERAVKDELLSEKPEVK	47-64
p34	YFK	36-38
(NF1-L)	EEERAVKDELLSEKPEVK	47-64
	DIRPEYREDFVL	78-89
p33	YFK	41-43
(NF1/RED1)	DEERAVKDELLSEKPEXK	52-69
	XPXXTNPALXVQPH	154-167

Fig. 2. **SDS-polyacrylamide gel electrophoresis of purified SF-A and partial amino acid sequences of SF-A.** (A) SF-A was separated into several proteins by electrophoresis, and the major components (p41, p39, p34, and p33) are indicated (lane 2). Size marker proteins are also shown (lane 1). Purified SF-A proteins were analyzed by immunoblot by using the anti-NFI-A antibody (lane 3). (B) The amino acid sequences of p41, p39, p34, and p33 were determined. Numbers correspond to the position of the amino acid sequences predicted from NF1-L cDNA (*10*) or NF1/RED1 cDNA (*11*).

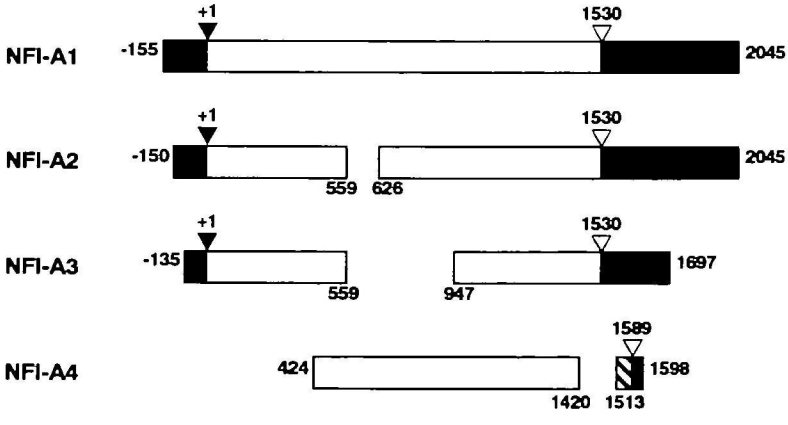


Fig. 3. **Schematic representation of the NFI-A clones.** Base positions are numbered according to that of NFI-A1, and position +1 is the A residue in the start codon. Open boxes represent identical sequences of the open reading frames among NFI-A isoforms. The frame-shifted region in NFI-A4 is marked by a slanting striped box. Filled boxes indicate 5′- and 3′-untranslated regions. Filled and open triangles represent start and stop points, respectively.

Almost all the proteins of the affinity-purified SF-A reacted with the anti-NFI-A antibody. Of these, three major proteins, p41, p39, and p34, were strongly reacted, whereas the other major protein, p33 derived from NFI-B, was stained weakly, probably due to the cross-reaction.

In the case of rat, only NF1-L/NFI-A cDNA has been cloned and reported, although the amino terminal region of NF1-L cDNA was not identified (*10*). A comparison of isoforms derived from orthologous NF1 genes revealed that their sequences were highly conserved during evolution, displaying similarities of up to 98% in the amino acid identities (*20*).

Isolation of Multiple NFI-A cDNAs Containing Alternative Coding Regions—In order to isolate the full-length NFI-A cDNA and other isoforms of rat NF1 family cDNA, a rat liver cDNA library was screened. We used a mouse NFI-A, originally termed NFI-B1 (*14*), as a probe, simply because NF1 clones were known to be homologous to each other. Then, using the newly isolated cDNA as a probe, we further obtained 32 independent clones from another rat liver cDNA library. The nucleotide sequences revealed that the obtained clones could be classified into four types: NFI-A1, NFI-A2, NFI-A3, and NFI-A4 (Fig. 3). NFI-A1 had no deleted region compared with NF1-L cDNA, but the regions 560-625 and 560-946 were deleted by alternative splicing from NFI-A2 and NFI-A3, respectively, leaving the protein coding frame unchanged. In contrast, NFI-A4 lacked a part of the common sequence (1421-1512) and was shifted by one frame. All the obtained clones were derived from the NFI-A gene. λ61 and λ58 belonged to NFI-A2 and NFI-A3, respectively, and seven independent clones were NFI-A4. The other 23 clones were derived from NFI-A1. λ68 (NFI-A1) seems to contain the complete open reading frame of NFI-A, and the nucleotide sequence of the overlapping region of NFI-A1 is identical to that of the partial NF1-L cDNA (1,712 bp) reported previously (Fig. 4). The four NFI-A isoforms isolated here included the three peptides, p41, p39, and p34, described above. Therefore,

we could not define which peptide derives from which isoform, although NFI-A1 and NFI-A4 are dominant forms in the normal rat liver, as described below.

Expression of Alternatively Spliced Variants of NFI-A mRNAs—To determine the mRNA expression of the four kinds of NFI-A clones in rat liver, reverse transcriptase-coupled PCR was carried out with several sets of primers spanning the deleted regions of NFI-A2, NFI-A3, and

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                                -155 ccgagttggaaatgtgaacgcaagaagcaggcttgatttttttttcctccccccctctcctagctc  -91
          tctcctcctctctctccctctcgcacccacactcacgcacacctccagcccgcacacagacgtgcacgcacccccgccgcccggcagtt   -1
          atgtattctccgctctgtctcacccaggatgagtttcatcctttcattgaagcacttctgccccatgtccgcgccttcgcctacacatgg   90
(NFI-A1)  M  Y  S  P  L  C  L  T  Q  D  E  F  H  P  F  I  E  A  L  L  P  H  V  R  A  F  A  Y  T  W    30

          ttcaacctgcaggcccgaaagcggaaatacttcaaaaagcatgagaagcgcatgtcgaaagaagaggagagggctgtgaaggatgaactg   180
(NFI-A1)  F  N  L  Q  A  R  K  R  K  Y  F  K  K  H  E  K  R  M  S  K  E  E  E  R  A  V  K  D  E  L    60

          ctaagtgagaagcctgaggtcaagcaaaagtgggcttcccgacttctggccaagttacggaaagatatccgacccgagtaccgagaggat   270
(NFI-A1)  L  S  E  K  P  E  V  K  Q  K  W  A  S  R  L  L  A  K  L  R  K  D  I  R  P  E  Y  R  E  D    90

          tttgttcttacagttacagggaaaaaacctccatgctgtgttctttccaacccagaccagaaaggcaagatgcggagaattgactgcctc   360
(NFI-A1)  F  V  L  T  V  T  G  K  K  P  P  C  C  V  L  S  N  P  D  Q  K  G  K  M  R  R  I  D  C  L   120

          cgccaggcagataaagtatggaggttggacctcgtcatggtgatcttgttcaaaggtattccgctggaaagtactgatggtgaacgcctt   450
(NFI-A1)  R  Q  A  D  K  V  W  R  L  D  L  V  M  V  I  L  F  K  G  I  P  L  E  S  T  D  G  E  R  L   150
(NFI-A4)                                                                    -  -  -  -  -  -  -  -  -

          gtgaagtccccacagtgctcgaatccagggctctgtgtccagccccatcacatagggggtttctgtaaaggaactcgatttatatttggca   540
(NFI-A1)  V  K  S  P  Q  C  S  N  P  G  L  C  V  Q  P  H  H  I  G  V  S  V  K  E  L  D  L  Y  L  A   180
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          tactttgtacatgcagcagattcaagtcaatctgaaagtcccagccaaccaagtgatgctgacattaaggaccagccagaaaatggacat   630
(NFI-A1)  Y  F  V  H  A  A  D  S  S  Q  S  E  S  P  S  Q  P  S  D  A  D  I  K  D  Q  P  E  N  G  H   210
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ttgggcttccaggacagcttcgtcacatcaggtgttttcagtgtgactgagctagtaagagtgtcacaaacaccaatagctgcaggaacc   720
(NFI-A1)  L  G  F  Q  D  S  F  V  T  S  G  V  F  S  V  T  E  L  V  R  V  S  Q  T  P  I  A  A  G  T   240
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ggccccaatttttctctctctgatttggaaagttcctcatactacagcatgagtccaggagcaatgaggaggtctctacccagcacatcc   810
(NFI-A1)  G  P  N  F  S  L  S  D  L  E  S  S  S  Y  Y  S  M  S  P  G  A  M  R  R  S  L  P  S  T  S   270
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          tctaccagctctaccaagcgcctcaagtctgtggaggacgaaatggacagtcctggtgaagaaccattttacacaggccaagggcgctcc   900
(NFI-A1)  S  T  S  S  T  K  R  L  K  S  V  E  D  E  M  D  S  P  G  E  E  P  F  Y  T  G  Q  G  R  S   300
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ccagggagtggcagccagtccagcggatggcatgaagtagagccaggcatgccatctccaaccactctgaagaagtctgagaagtctggt   990
(NFI-A1)  P  G  S  G  S  Q  S  S  G  W  H  E  V  E  P  G  M  P  S  P  T  T  L  K  K  S  E  K  S  G   330
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ttcagcagcccctcccccttcgcagacctcctccctcggaacagcattcacacagcatcaccgacctgtcataacaggaccgagagcaagc  1080
(NFI-A1)  F  S  S  P  S  P  S  Q  T  S  S  L  G  T  A  F  T  Q  H  H  R  P  V  I  T  G  P  R  A  S   360
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ccacatgcgacgccatcgactctccactttccaacgtcacctatcatccagcagcctgggccttatttctcacacccagccatccgttac  1170
(NFI-A1)  P  H  A  T  P  S  T  L  H  F  P  T  S  P  I  I  Q  Q  P  G  P  Y  F  S  H  P  A  I  R  Y   390
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          caccctcaggagacgctgaaggagtttgtccaacttgtctgccccgatgctggtcagcaagctggacaggtggggttcctcaatcccaat  1260
(NFI-A1)  H  P  Q  E  T  L  K  E  F  V  Q  L  V  C  P  D  A  G  Q  Q  A  G  Q  V  G  F  L  N  P  N   420
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ggaagcagtcaaggcaaggtgcacaacccattcctccccaccccaatgttgccgccgccaccgccaccaccgatggccaggcctgtgcct  1350
(NFI-A1)  G  S  S  Q  G  K  V  H  N  P  F  L  P  T  P  M  L  P  P  P  P  P  P  P  M  A  R  P  V  P   450
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          ctgcccatgccagacaccaagcctccaaccacatcaacagaaggcggtgcagcctcccccacctcaccgacctactcgacacccagcacc  1440
(NFI-A1)  L  P  M  P  D  T  K  P  P  T  T  S  T  E  G  G  A  A  S  P  T  S  P  T  Y  S  T  P  S  T   480
(NFI-A4)  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  I/
          tcccccgcaaaccgattcgtcagtgttggaccacgggatccaagctttgtaaatatccctcaacagacacagtcctggtacctgggataa  1530
(NFI-A1)  S  P  A  N  R  F  V  S  V  G  P  R  D  P  S  F  V  N  I  P  Q  Q  T  Q  S  W  Y  L  G  *   509
(NFI-A4)                                                                    /  L  V  P  G  I  K
          aagttgcagcgtcccaccatcctccagacagaccacctgacccttctcaactctgtaacatggacgcaacctcaacccggcgcagttac  1620
(NFI-A4)    V  A  A  S  H  H  P  P  D  R  P  P  D  P  F  S  T  L  *
          aacttcactgtcagtggaaggggagagacaccaactcgaatcaacttgtacatggaaacagcgaacgtcgtggtcaaacagcaaaggcca  1710
          taacttttcgggattcttttttttttttaatactttagggactgtcgtaatttctcatatggtgctggaaatggttgggctgtgtgaca  1800
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          gagcctggccttttcacagtatttcaagaatttgcccgcgcaggtgtgtgaccctcccggagcacttggagacacctggagaatgaaatc  1980
          tttcgtagtagctaagatctgtgatctgtaacttacaggacactcaaagggcaatgtttttggg                          2045
```

Fig. 4. **Nucleotide and deduced amino acid sequences of NFI-A1 and NFI-A4.** Nucleotides and amino acids are numbered on the right. Position +1 is the A residue or the methionine residue of the translational start codon. Amino acid sequences corresponding to partial amino acid sequences derived from p41, p39, and p34 are underlined.

NFI-A4 (Fig. 5). The amplified PCR products were subcloned and sequenced. With the primer pairs S-88/S-95 and S-88/S-99, including the deleted regions of NFI-A2, and NFI-A3, respectively, only one band of a size corresponding to NFI-A1 could be amplified, whereas use of the primer pair S-66/S-64 resulted in two PCR products. The major product was derived from NFI-A1, and the minor one corresponded to NFI-A4. These results indicate that NFI-A1 is expressed at higher levels than NFI-A4, and NFI-A2 and NFI-A3 are rarely detected in the rat liver. When the rat liver cDNA library was screened, 23, 1, 1, and 7 clones of NFI-A1, NFI-A2, NFI-A3, and NFI-A4 were obtained, respectively. The expression profile of NFI-A derived from the reverse transcription-PCR using rat liver total RNA was coincident with the number of clones from the rat liver cDNA library.

Binding of NFI-A to SF-A Sites of the GST-P Silencer and Promoter—Partially purified SF-A binds to the multiple *cis*-elements (GPS0A, GPS0B, GPS3, GPS4, GPS5, and site7) in the silencer and the promoter in the GST-P gene (Fig. 1). The binding activity of SF-A to GPS4 is higher than that of other binding sites (*7*). We performed DNase I footprint analysis to compare the DNA binding activities of partially purified SF-A and recombinant NFI-A protein (Fig. 6). For this purpose, we used a common DNA-binding region in NFI-A1 and NFI-A4, because NFI-A1 and NFI-A4 are dominant forms of NF1 proteins in rat liver. Recombinant NFI-A bound to other SF-A binding sites as well as to GPS4, and bound to GPS4 more strongly than other binding sites, as also observed with purified SF-A. Thus the protection profiles of the two proteins were indistinguishable.

Binding Activity of NFI-A to GPS4 in the Rat Liver Nuclear Extracts—The NF1 site for adenovirus replication origin (NF1-adeno) is a high-affinity binding site of NFI-A (*21*). For characterization of the binding factor to GPS4 in the rat liver nuclear extracts, we performed a gel shift assay using several competitors and an antiserum raised against the DNA-binding domain of NFI-A (*13*). Rat liver nuclear extracts and labeled GPS4 were incubated with or without the specific and non-specific competitors, and

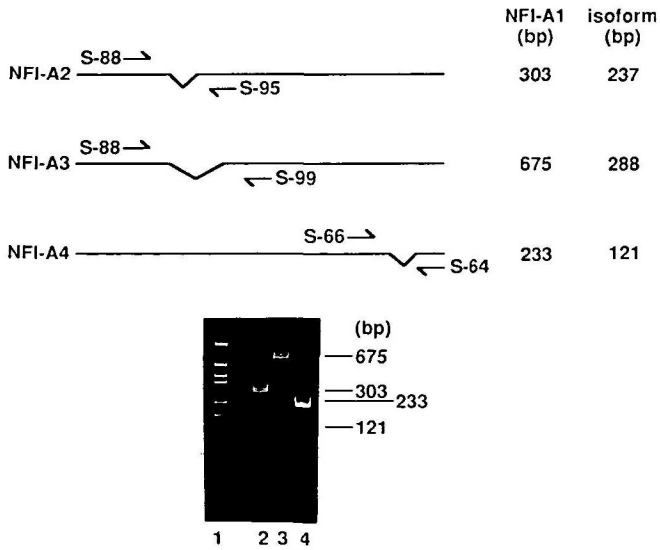

Fig. 5. **Expression profile of NFI-A splicing variants in the rat liver.** Reverse transcriptase-coupled PCR analysis of rat liver RNA using different sets of PCR primers spanning the spliced-out regions. Upper panel shows the location of PCR primers and the predicted sizes of the products derived from NFI-A1 and splicing variants. Lower panel indicates the gel electrophoretic pattern of PCR products. Primers pair are S-88 and S-95 (lane 2), S-88 and S-99 (lane 3), and S-66 and S-64 (lane 4), respectively. pBluescript digested with *Hpa*II was used as a size marker (lane 1).

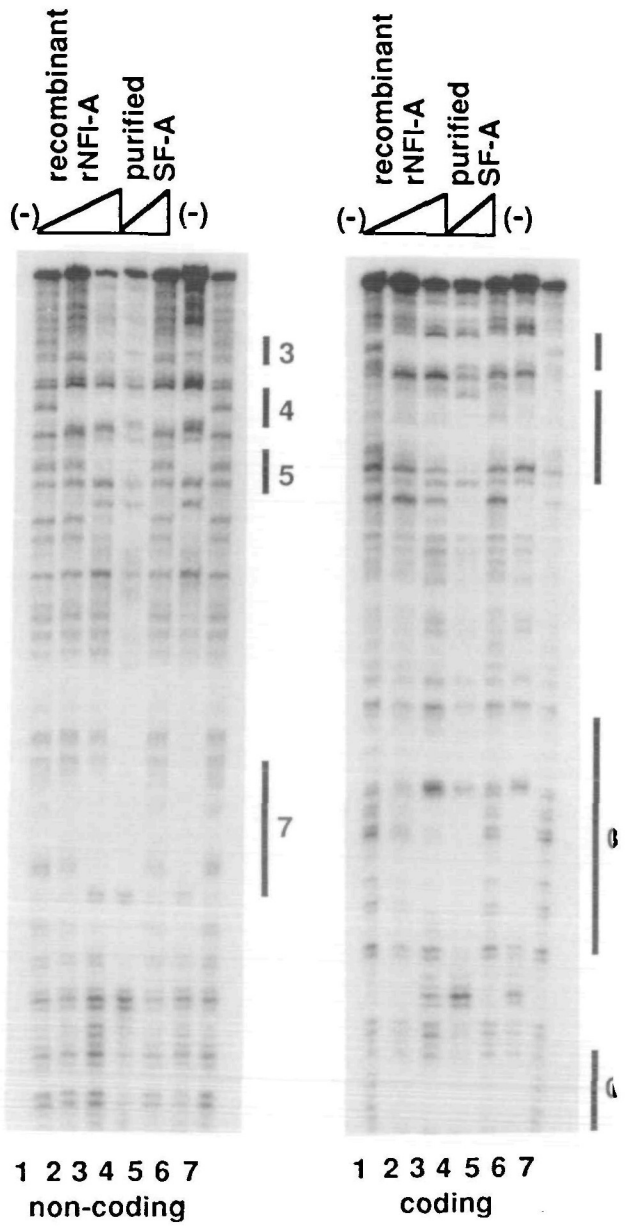

Fig. 6. **DNase I footprint analysis of recombinant NFI-A and purified SF-A.** A *Bam*HI-*Hin*dIII fragment corresponding to −396 to +59 in the GST-P gene was labeled at the *Hin*dIII end of the non-coding strand (left panel), and a *Hin*dIII-*Sac*I (−396 to −90) fragment was labeled at the *Hin*dIII end of the coding strand (right panel). As a control, BSA was added in lanes 1 and 7 indicated as (−). Increasing amounts of recombinant NFI-A (0.125, 0.25, and 0.5 μl) and purified SF-A (1 and 3 μl) were used for the detection of the binding sites (lanes 2 to 6). The several regions protected from DNase I digestion by recombinant NFI-A and purified SF-A binding are indicated by vertical bars.

DNA/protein complexes were separated on native polyacrylamide gel (Fig. 7). Both of the shifted bands disappeared after the addition of the specific competitor NF1-adeno. Binding activity was reduced depending on the input amount of GPS4, while GPS1, the C/EBP family protein binding site, had no effect on the mobility of these complexes. Since NF1-adeno was a more effective competitor than GPS4, the binding affinity of the binding protein in the nuclear extracts to NF1-adeno was thought to be higher than that of GPS4 (Fig. 7, lanes 3-6). To characterize the GPS4 binding proteins, we performed a gel shift assay using an antibody against the NFI-A DNA-binding domain. The major and faster migration complex was super-shifted strongly by the addition of the antibody, whereas the preimmune serum caused no changes of pattern. The minor and slower complex reacted very weakly with the antibody compared with the control lane, although the composition of this band has not yet been characterized in detail.

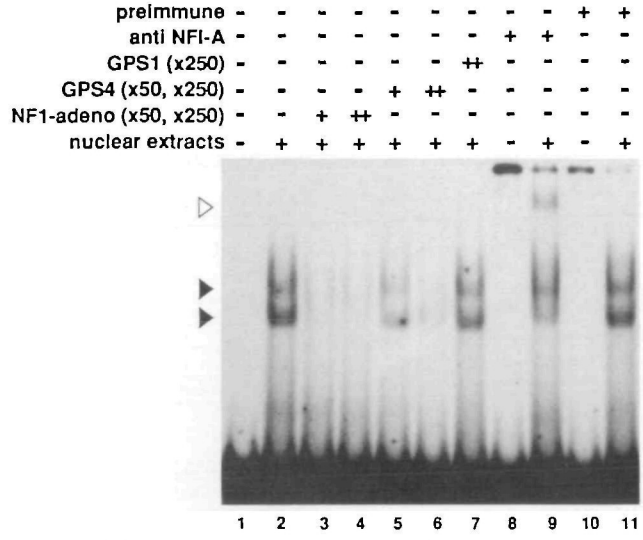

Fig. 7. **GPS4 formed complexes with NFI-A in rat liver nuclear extracts.** Gel shift analysis of the rat liver nuclear extracts was performed with GPS4 as a probe with or without NFI-A antibody. NF1-adeno, GPS4, or GPS1 was used as the competitor. Lane 1, probe only; lane 2, control (without competitor); lane 3 and lane 4, specific competitor NF1-adeno was used at 50- and 250-fold molar excess, respectively; lane 5 and lane 6, specific competitor GPS4 was used at 50- and 250-fold molar excess, respectively; lane 7, competitor GPS1 as another transcription factor binding site was used at 250-fold molar excess; lanes 9 and 11, nuclear extracts plus NFI-A antibody and preimmune serum, respectively; lanes 8 and 10, probe plus NFI-A antibody and preimmune serum, respectively, without nuclear extracts. Closed and open triangles indicate the specific DNA/protein complexes and super-shifted complexes, respectively.

Repression of Transcription Mediated by GAL4-NFI-A—Since NF1 proteins were expressed in almost all culture cells, we fused the carboxy terminal of NFI-A proteins to the DNA-binding domain of GAL4 (1-147) in order to exclude the effect of endogenous NF1 proteins on the transcription. The expression vectors were cotransfected into HepG2 cells with a luciferase reporter gene which contains the human metallothionein II_A ($hMTII_A$) promoter element having five binding sites for the GAL4 DNA-binding domain (Fig. 8). The $hMTII_A$ promoter exhibited a high level of transcriptional activity when cotransfected with the expression vector including the GAL4 DNA-binding domain alone, because it contains basal level enhancers. However, the transcription activities were repressed when cotransfected with the GAL4-NFI-As as expression vectors. Differences in repression activities were indistinguishable among GAL4-NFI-As. Thus, the carboxy terminal of NFI-A can function as a repressor of transcription in a DNA binding site-dependent manner.

DISCUSSION

SF-A binds to several regions in the silencer of the GST-P gene and plays an important role in the negative regulation of this gene (*7*). Purified SF-A from rat liver nuclear extracts was separated on SDS-polyacrylamide gel and consisted of several proteins (60-30 kDa species). Determination of the microsequences of the major components (p41, p39, p34, and p33) of SF-A revealed that these proteins are identical to NF1-L and NF1/Red1, members of the NF1 family (*10, 11*). Kruse *et al.* reported that the chicken NF1 proteins are encoded by at least four independent NF1 genes (NFI-A, NFI-B, NFI-C, and NFI-X) (*20*). In the case of rat, only NF1-L cDNA encoded by the NFI-A gene has been cloned. In the present study, partial amino acid sequences of p33 were found to be identical to the

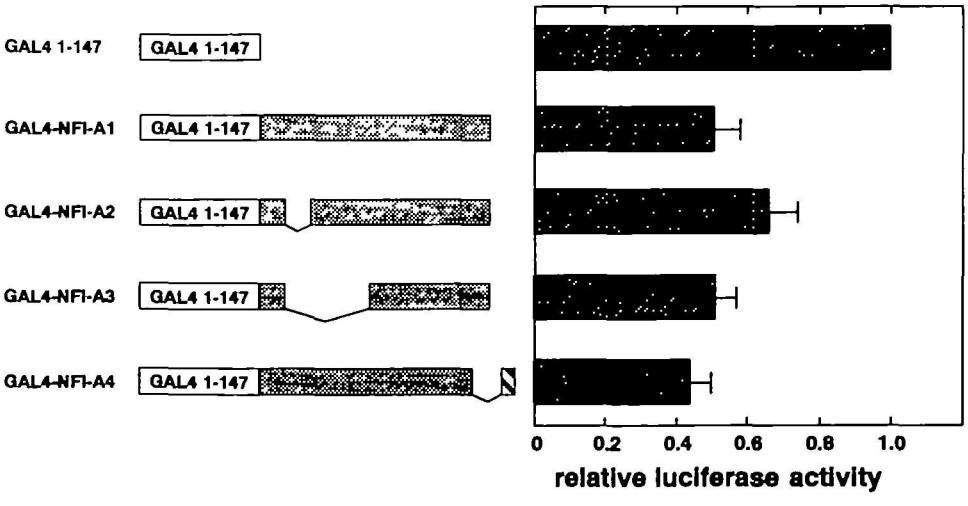

Fig. 8. **Repression activity of the carboxy terminal domains from NFI-A splicing variants.** The left panel shows the effector plasmids which contain the GAL4 DNA-binding domain and the C-terminal region of various NFI-As. Each dish received the reporter plasmid containing GAL4 binding sites and β-galactosidase expression vector as well as the effector plasmid. Luciferase activity was measured in cell extracts, and normalized by the β-galactosidase activity. The right panel shows the values presented as relative luciferase activity $\pm$standard deviation (n=13-16) relative to the activity of cotransfection of the GAL4 DNA-binding domain alone.

sequence of NF1/Red1 derived from hamster NFI-B gene, indicating that NFI-B gene and its product exist in the rat. NFI-A antibody reacted with almost all the proteins of affinity purified SF-A, but weakly cross-reacted with p33 (NFI-B). p50 and p60 are abundant proteins in purified SF-A, after the major components. p60, which was recognized by NFI-A antibody more efficiently than p50, may be undegraded NFI-A, and p50 may be a different member of the NF1 family (Fig. 2A, lane 3).

Several splicing isoforms of NF1 have been cloned from human, chicken, and mouse, but not rat (*14, 20, 22, 23*). In the present study, we isolated three splicing isoforms derived from rat NFI-A gene.

The regions, 560-625 and 560-946, are spliced out from NFI-A2 and NFI-A3, respectively, leaving the protein coding frame unchanged. Another isoform, NFI-A4, lacked a part of the common sequence (1421-1512), and was shifted by one frame. Several lines of evidence suggest that it is unlikely that these isoforms are artifacts derived from library construction. First, we have recently isolated the NFI-A gene of the rat, comprising a total of 11 exons, and determined the splice sites of the gene (unpublished results). All the sequences of the exon/intron boundary satisfy the GT-AG rule. These sequence analyses revealed that the regions corresponding to exon 3, exon 3-6, and exon 10, were spliced out from NFI-A2, NFI-A3, and NFI-A4, respectively. Secondly, the deleted region of NFI-A2 was identical to that of chicken NFI-A3 (*20*). Moreover, chicken NFI-A1 isoform has an insertion at the site of exon 6 (*20*). These indicate that cloned NFI-A2 and NFI-A3 are not artifacts derived from library construction, although they are rarely expressed and not detected by reverse transcriptase-coupled PCR in the rat liver.

NF1 was originally identified as a nuclear factor that binds to the replication origin of adenovirus and initiates DNA replication *in vitro* (*24*), and has been shown to activate transcription from the promoters of the human α-globin, human papillomavirus type 16, and mouse myelin basic protein (*14, 25, 26*). However, NF1 binds to the negative regulatory element in the rat growth hormone gene, human retinol binding protein, rat peripherin gene, and chicken cartilage matrix protein gene (*27-31*). NF1 products from the four NF1 genes and their spliced isoforms, which are highly conserved at the amino-terminal DNA-binding domain and heterogeneous at the carboxy-terminal domain, raise the possibility that different NF1 proteins may possess different functions. *Drosophila* SL-2 cells, which do not express endogenous NF1 proteins, have been used to observe the transcriptional regulation of NF1. CTF/NF1, human NFI-C gene product, and human NFI-X act as transcriptional activators in SL-2 cells (*22, 23*). It has not been determined whether NFI-A and NFI-B have effects on the transcription in SL-2 cells.

Though NF1 binding sites have been isolated as positive and negative regulatory elements, a transcriptional repression domain has not yet been identified from NF1 family proteins. In the present study, the ability of NFI-A has been demonstrated to repress transcription as GAL4 fusion proteins. In addition, all of the NFI-A splicing variants showed the same repression activity. The proline rich region of NFI-C functions as a transcriptional activation domain. In contrast, WT1, a Wilms tumor gene product, contains a transferable repressing domain that is rich in proline residues (*32*). Although the repression domain has not yet been characterized, the proline rich domain (amino acid 430-459) exists in the carboxy terminus of all NFI-A isoforms. Therefore, the repression activity might be mediated by the proline rich domain of NFI-A.

The results of the present SF-A purification indicate that NFI-A is a dominant form of the NF1 proteins, and NFI-B might be expressed at higher levels than NFI-C and NFI-X in the rat liver. Although the function of NFI-B is unclear, it is likely that NFI-A occupies SF-A binding sites including GPS4 and represses the GST-P gene expression in the rat liver.

One major and one minor complex were detected on the gel shift assay using rat liver nuclear extracts and GPS4 as a probe (Fig. 7). The slower migration complex reacted very weakly with NFI-A antibody. Therefore, the unidentified proteins may be involved in the minor complex, although we have not clarified this component, and also cannot rule out the possibility that the difference in migration derived from full-length NF1 and spliced isoforms, or degradation products. For the maximum silencer activity, all the DNA-binding sites of SF-A, SF-B, and SF-C are required (*7*). To further investigate the negative regulation mechanism of the GST-P gene expression, we are in the process of the cloning the SF-C cDNA, and also are examining the characteristics of the functional interactions of SF-A (NF1 family) and SF-B (C/EBP family) *in vivo* and *in vitro*.

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