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Transcriptional Enhancement of UDP-Glucuronosyltransferase Form 1A2 (UGT1A2) by Nuclear Factor I-A (NFI-A) in Rat Hepatocytes

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In cultured primary hepatocytes UDP-glucuronosyltransferase form 1A2 (UGT1A2) mRNA level is 80 times higher than that found in rat liver. We previously identified an enhancer sequence in the UGT1A2 promoter, and designated it as culture-associated expression responsive enhancer module (CEREM). Affinity chromatography with DNA fragments containing CEREM allowed enrichment of nuclear factor I (NFI) proteins from cultured hepatocytes. The NFI family is encoded by four distinct genes, NFI-A, NFI-B, NFI-C, and NFI-X. Immunoblot analysis with isoform-specific antibodies showed that NFI-A1 existed as a major component in rat liver and cultured hepatocytes. By contrast, NFI-C1 was present in rat liver but disappeared immediately upon cultivation of hepatocytes. Only trace amounts of NFI-B and NFI-X were detectable in rat liver and cultured hepatocytes. NFI-A1 elevated expression of the reporter gene that is under the control of CEREM, while NFI-C1 had an inhibitory effect. Co-expression of a constant amount of NFI-A1 with an increasing amount of NFI-C1 led to a concentration-dependent decrease in the expression of the CEREM-controlled reporter gene mediated by NFI-A1. Activation of UGT1A2 expression by NFI-A1 is suppressed by the coexistence of NFI-C1 in the liver, and culture-associated expression of UGT1A2 is triggered by the rapid disappearance of NFI-C1 in cultured hepatocytes.

Key words: drug-metabolizing enzyme, enhancer, hepatocyte, transcriptional regulation, UDP-glucuronosyltransferase.

Abbreviations: UGT, UDP-glucuronosyltransferase; CEREM, culture-associated expression responsive enhancer module; NFI, nuclear factor I; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; PBS, phosphate-buffered saline; HA, hemagglutinin; GST, glutathione *S*-transferase; CYP, cytochrome P-450; PBRU, phenobarbital-responsive enhancer unit; CAR, constitutive androstane receptor; RXR, retinoid X receptor.

Liver-specific functions, such as drug metabolism and lipid catabolism, are regulated qualitatively and quantitatively by a variety of endogenous and exogenous factors including hormones, nutritional conditions, and foreign chemicals. Glucuronidation is a major enzymatic pathway that facilitates the biotransformation of many lipophilic xenobiotics and endobiotics into more water-soluble compounds. This is considered to be detoxification, because the resulting glucuronidated metabolites exhibit less biological or chemical activity than the parent materials and are rapidly excreted into the bile or urine. UDP-glucuronosyltransferase (UGT) comprises a family of phase II drug-metabolizing enzymes that catalyze glucuronidation (*1*). On the basis of sequence similarity, UGTs can be classified into two subfamilies, UGT1 and UGT2, which comprise drug-glucuronidating and steroid-glucuronidating forms, respectively (*2*). Characterization of genomic DNA clones encoding the *UGT1* gene has shown that the *UGT1* locus comprises multiple first exons that encode isoform-specific sequences and a single set of commonly used exons (exons II, III, IV, and V) that encode the same sequence for all UGT1 isoforms (*3–5*). Each UGT1 isoform arises from the complex gene through the combination of a unique first exon and the commonly used exons. Transcription from the unique first exon is controlled by a corresponding promoter.

UGT1A2[1] is a member of the UGT1 family and exhibits a unique pattern of gene regulation in contrast to the other UGT1 isoforms. In a previous paper, we demonstrated that UGT1A2 is highly induced in hepatocyte primary cultures, while expression of UGT1A2 is very low in rat liver (*6*). When hepatocytes are isolated from liver and transferred to primary cultures, the composition of liver-specific proteins in the cultured cells is significantly altered. For example, the cellular content of cytochrome P-450 (CYP) in hepatocytes rapidly decreases within a few days under conventional primary culture conditions (*7–9*). The reduction of such liver-specific proteins is thought to be mainly due to a transcriptional decrease

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[1]The UGT1 gene is a single locus with multiple first exons followed by one set of commonly used exons (II, III, IV, and V), and each UGT1 isoform is generated through alternative splicing (*3–5*). We tentatively divided the UGT1 isoforms into a phenol cluster (cluster A) and a bilirubin cluster (cluster B) according to their sequence homology and preferential substrates (*5*). According to the recommended nomenclature system proposed by Mackenzie *et al.* (*2*), the number of each UGT1 isoform indicates the exact location of the first exon from the commonly used exons in the UGT1 locus. Thus, UGT1A2 is formed by combination of exon A2 with the common exons.

(*7*). On the other hand, the expression of some proteins, such as heme oxygenase (*9*, *10*) and P-glycoprotein (*11*), is markedly higher in primary hepatocyte cultures than in the liver. Several groups have reported that cultured adult rat hepatocytes show fetal phenotypic expression of several proteins (*9*, *12*, *13*). In prolonged hepatocyte cultures, there is gradual "fetalization," that is adult-to-fetal transition, of the expression of specific proteins such as aldolase isozyme A, γ-glutamyl transpeptidase, and α-fetoprotein. In spite of these alterations in liver-specific proteins in association with cultivation of hepatocytes, primary cultures of hepatocytes are widely used to investigate various liver functions under conditions free from physiological regulation. However, the molecular mechanisms involved in these alterations have been poorly characterized.

UGT1A2 mRNA is not present in fetal rat livers according to the results of RT-PCR analysis (Y. Emi, M. Kishi, S. Ikushiro, and T. Iyanagi, unpublished results). This observation thus suggests that induction of UGT1A2 is not attributable to the fetalization of cultured hepatocytes, but is likely to be controlled through distinct transcriptional regulation. We have observed that a 66-bp enhancer module is located between –307 and –242 upstream of the transcription start site, and is required for the culture-associated induction of UGT1A2. This enhancer module was designated as culture-associated expression responsive enhancer module (CEREM). Our previous studies revealed a specific protein binding to CEREM in the nuclei of cultured hepatocytes (*6*). DNase I protection analysis and methylation interference assay revealed the existence of a 5′-CTGGCAC-3′ core sequence in CEREM, which resembles a half-site of the nuclear factor I (NFI)–binding motif (*14*). CEREM contains two additional motifs, one is a characteristic repetitive sequence and the other a consensus sequence for activator protein-1 (*15*).

To gain an insight into the mechanism of regulation of the unique UGT1A2 expression, we focused on the CEREM region that is sufficient for the culture-associated transcriptional enhancement of the heterologous promoter in primary hepatocytes (*6*). We purified CEREM-binding proteins from cultured hepatocytes by DNA-affinity chromatography. Characterization of the purified CEREM-binding proteins revealed that the most abundant 30-kDa protein is an isoform of the nuclear factor I (NFI) family. The NFI family comprises site-specific DNA-binding proteins which recognize a palindromic consensus sequence, 5′-TGGCA$(N)_3$TGCCA-3′, as homodimers and heterodimers through an amino terminal DNA-binding and dimerization domain (*16*). The NFI family is encoded by four distinct but closely related genes, NFI-A, NFI-B, NFI-C, and NFI-X, in vertebrates (*17–23*). In addition, the diversity of the NFI protein family (430–560 aa) can be generated through alternative splicing of primary transcripts derived from individual NFI genes to yield many different mRNAs (*24*). However, the detailed molecular basis of the functional differences between these divergent forms remains unclear. NFIs regulate the transcription of a large variety of cellular and viral genes, and the NFI genes are expressed in many different tissues, suggesting that NFIs play a crucial role in the cellular functions of many organs (*21*, *25–30*). Upon binding to NFI recognition sites, NFIs can either up-regulate or down-regulate transcription from the corresponding promoters, however, the precise molecular mechanism of transcriptional activation and repression by NFIs remains unknown (*16*). In this study we further isolated 12 cDNA species encoding distinct NFI isoforms from rat hepatocytes, and analyzed the expression profiles of both NFI mRNAs and NFI proteins. Through co-transfection of human hepatocarcinoma HepG2 cells with each of the 12 NFI isoforms together with a reporter gene, we also confirmed which of the NFI isoforms participated in the culture-associated transcriptional enhancement of the UGT1A2 gene.

EXPERIMENTAL PROCEDURES

Primary Cultures of Rat Hepatocytes and Preparation of Nuclear Extracts—Male Wistar rats weighing 180–200 g were used in all experiments and fed *ad libitum* with commercial rat chow. They were kept in a temperature-controlled room with a 12-h light/dark cycle. Isolated hepatocytes were prepared by the two-step collagenase perfusion method as described previously (*6*, *31*). Freshly isolated hepatocytes were suspended at 5×10^5 cells/ml in Williams' medium E supplemented with 5% calf serum (GIBCO BRL, Gaithersburg, MD), 10^{-7} M dexamethasone, 10^{-7} M insulin, and 30 μg/ml kanamycin. 10-ml portions of the cell suspension were plated onto 10-cm culture dishes coated with type I collagen. The culture medium was changed two hours after plating, and then daily thereafter.

Nuclear extracts were prepared from primary cultures of rat hepatocytes as previously described (*6*, *31*). Rat liver nuclear extracts were prepared essentially as described by Frain *et al*. (*32*). Nuclear extracts were dialyzed against a buffer comprising 20 mM Hepes-KOH (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, and 0.2 mM PMSF. The gel mobility shift assay was performed as previously described (*31*). In antibody supershift experiments, nuclear extracts were preincubated at 4°C with specific antibodies for 60 min before being incubated with probe DNA. DNA–protein complexes were separated on a 5% polyacrylamide gel with a buffer comprising 40 mM Tris-HCl (pH 8.5), 380 mM glycine, and 0.2 mM EDTA, and were visualized with a FLA3000 image analyzer (Fuji, Tokyo, Japan).

Purification of the CEREM-Binding Protein and Sequencing of Its NH_2-Terminal—A biotinylated DNA fragment (between –307 and –242) was amplified by polymerase chain reaction (PCR). The primers used for the PCR were as follows:

F307: 5′-ACCTTCAAATCGTGTTCCCTGATC-3′
R242: 5′-CTTTGCCATGACTCATACTCTTC-3′

R242 was conjugated with biotin at the 5′ end. The resultant biotinylated DNA fragment was purified by gel electrophoresis and then mixed with TetraLink™ avidin resin (Promega, Madison, WI). The resulting DNA-conjugated resin was packed into a mini column and used for DNA-affinity chromatography.

The dialyzed crude nuclear extracts were centrifuged at 15,000 × *g* for 20 min at 4°C. Buffer A, which was the basal buffer used in the following purification steps, consisted of 20 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA,

10% glycerol, 0.5 mM DTT, 0.05% Triton X-100, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The clear supernatant was applied to a DE52 column equilibrated with buffer A containing 50 mM KCl. The DE52 column was washed with buffer A containing 50 mM KCl and eluted with buffer A containing 0.2 M KCl. Fractions containing CEREM-binding activity were pooled, diluted 1:1 with buffer A, and then mixed with poly (dI-dC) to a final concentration of 40 μg/ml. After incubation on ice for 15 min, the mixture was loaded onto a DNA-affinity column. The affinity column was washed with buffer A containing 0.1 M NaCl and eluted with buffer A containing 0.6 M NaCl. The active fractions were pooled and diluted again with buffer A to 0.1 M NaCl, and then they were subjected to further cycles on the same DNA-affinity column. After three cycles through the DNA-affinity column, a 100-μl aliquot of the eluate was precipitated with five volumes of acetone, centrifuged, and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a PVDF membrane and stained with Coomassie Brilliant Blue. Protein bands were cut from the membrane, and then the NH_2-terminal sequences of the materials were analyzed with a Shimazu PSQ-1 protein sequencer (Kyoto, Japan). For the denaturation–renaturation experiment, ~20 μg of each purified fraction was precipitated as above, and then subjected to SDS-PAGE alongside 0.2 μg of the purified fraction used as a position marker. After the run, the marker lane was cut out and visualized by silver staining. The regions of the gel containing the desired protein bands were excised from the remaining sample lane, and the gel slices were processed as described (*33*).

Preparation of Total RNA, Isolation of NFI cDNAs, and Analysis of mRNA Expression—Total RNA was prepared from rat liver and primary hepatocytes by use of a QuickPrep™ Total RNA Extraction Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Cultured hepatocytes were washed twice with ice-cold phosphate-buffered saline (PBS), and then scraped from the dishes with a rubber policeman. Hepatocytes from two dishes (approximately 10^7 cells) were collected and processed to prepare total RNA. Briefly, the cell suspension was homogenized by 40 vigorous passages through an 18-gauge needle, and then mixed thoroughly with a CsTFA solution by shaking in a vortex mixer.

NFI family cDNA species were amplified by RT-PCR. Reverse transcription was carried out using SuperScript II (GIBCO BRL), and PCR was carried out using an LA-PCR kit (TaKaRa, Kyoto, Japan) as described in the instruction manual. The primers used for amplification were as follows (the *Eco*RI recognition sites are underlined):

IA-F: 5′-CGGAATTCCAGCCCGCACACAGACGTGC-3′
IA-R: 5′-CGGAATTCGCGTCCATGTTACAGAGTTGAGA-3′
IB-F: 5′-GCGAATTCATGATGTATTCTCCC-3′
IB-R: 5′-CGGAATTCTTTCCCCCTCAGTTGCTTGT-3′
IC-F: 5′-CGGAATTCGGGGATGTATTCCTCCCCGCT-3′
IC-R: 5′-CGGAATTCATTGCCATTGAGCTGGGAGTTG-3′
IX-F: 5′-CGGAATTCGATGTACTCCCCGTACTGCC-3′
IX-R: 5′-CGGAATTCTGTGGGATGTTCAGAAAGTTGC-3′

The IB-F and IC-F primer sequences were based on the orthologous sequences of hamster (*19*) and mouse (*21*), respectively, while the others were derived from rat NFI sequences (*34*, *35*). The amplified PCR products were treated with *Eco*RI and then ligated into pBluescriptII SK(+). The nucleotide sequences of the amplified cDNA fragments were confirmed by dideoxy sequencing with a PRISM 310 sequencer (Perkin-Elmer, Foster City, CA). The expression of mRNA was analyzed by PCR with reverse transcription (RT-PCR) as previously described (*5*, *6*). Primers for mouse glyceraldehyde-3-phosphate dehydrogenase were synthesized and used as standards for normalizing mRNA expression.

Preparation of Antibodies and Immunoblot Analysis of NFI Proteins—DNA fragments corresponding to amino acid residues 32–238 (NFI-A), 1–207 (NFI-B), 1–232 (NFI-C), and 140–400 (NFI-X) were excised from the pBluescript subclones, and separately recloned into pQE-30 series vectors (QIAGEN, Valencia, CA). Anti–rat NFI antibodies were raised in male rabbits against purified recombinant NFI proteins fused with a 6× His-tag at the NH_2-terminus. Immunoglobulin fractions were prepared from the pooled antisera by ammonium sulfate fractionation. Anti-NFI IgG (N20) was purchased from Santa Cruz Biotech., Inc. (CA, USA). N20 is a rabbit polyclonal antibody raised against the N-terminus of human origin, and recognizes all NFI isoforms of rat origin. Immunoblot analysis was performed as previously described (*36*). In particular gel shift experiments, NFI isoforms contained in the DNA-protein complex were analyzed by immunostaining of the shift band. After the electrophoresis, the gel was soaked in a buffer comprising 20 mM Tris base, 150 mM glycine, 20% methanol, and 0.1% SDS for 15 min at room temperature, and then electroblotted onto a nitrocellulose membrane. The membrane was further treated for immunostaining.

Construction of Plasmids and Transfection into Cultured Cells—In order to express NFI family proteins in cultured cells, the NFI cDNAs which had been subcloned into pBluescriptII SK(+) were excised by treatment with *Eco*RI and then recloned into the pUCDSRα vector (*37*). To generate hemagglutinin (HA)-tagged NFI proteins, the *Eco*RI-treated cDNA fragments were recloned into the pCMV-HA vector (CLONETECH, Palo Alto, CA). For construction of a reporter gene, a 66-base-pair DNA fragment containing CEREM (between –307 and –242) was amplified by PCR. The resultant PCR product was treated with T4 DNA polymerase to obtain blunt ends, and then phosphorylated with T4 polynucleotide kinase. This fragment was ligated into the pGL2-Promoter (Promega, Madison, WI), which had been cleaved with *Sma*I, to generate CR66/pGL2-P. The nucleotide sequence and the orientation of the inserted DNA fragment were confirmed by dideoxy sequencing.

COS-7 cells and HepG2 hepatocarcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO BRL). Cells were transfected using FuGENE6 Reagent (Roche, Indianapolis, IN) according to the supplier's protocol. Typically, in reporter assays the following DNA mixtures were added to 6-cm culture dishes; 0.9 μg of reporter plasmids for expression of firefly luciferase, 0.9 μg of SRα plasmids for expression of each NFI isoform, and 0.2 μg of

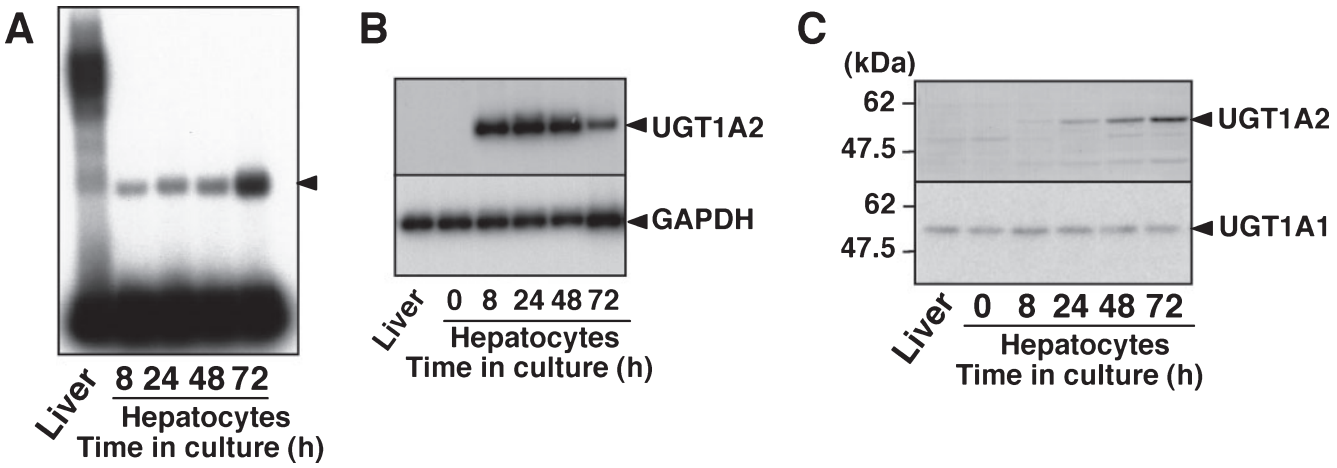


Fig. 1. **Time-dependent changes in UGT1A2 and CEREM-binding activity in primary cultures of rat hepatocytes.** (A) Detection of CEREM-binding proteins by gel mobility shift assay. The 66-base pair probe –307/–242 was generated by PCR, and the DNA probe was incubated with crude nuclear extracts prepared from rat liver and primary cultures of rat hepatocytes. The prominent shifted bands seen in liver and cultured hepatocytes are indicated by the closed arrowhead. (B) RT-PCR analyses of UGT1A2 mRNA. Hepatocytes were harvested at the indicated time points. Total RNA was prepared from rat liver and primary cultures of rat hepatocytes, and subjected to RT-PCR using UGT1A2-specific primer pairs. The ^{32}P-labeled PCR products were visualized by autoradiography. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed for normalization of this assay, and serves as a control for equal amounts of RNA input. (C) Detection of UGT1A2 proteins in primary cultures of rat hepatocytes by immunoblot analysis. Hepatocytes were harvested at the indicated time points. Microsomes were prepared from rat liver and cultured rat hepatocytes. The microsomal protein (15 μg) was analyzed by immunoblotting with UGT1A2-specific antibodies. Expression of UGT1A1 proteins is also shown at the bottom. The positions of standard molecular weight markers are indicated on the left.

pmiwZ (*38*) for expression of β-galactosidase as an internal control of transfection efficiency. Two micrograms of the mixed DNAs was treated with 6 μl of FuGENE6 reagent. Cells were incubated with the DNA-lipid complex for 40 h, washed with ice-cold phosphate-buffered saline, and then assayed for β-galactosidase and luciferase activities.

HepG2 cells, which express either the combination of NFI-A1/HA-tagged NFI-C1 or NFI-C1/HA-tagged NFI-A1, were used for immunoprecipitation. The collected cells (≈2 × 10^6 cells) were resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 10 mM NaCl, and 0.5% NP40), incubated for 30 min on ice, and then centrifuged for 5 min at 1,000 × *g*. The sedimented crude nuclear fractions were resuspended in 1 ml of ice-cold extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP40), incubated for 30 min on ice, and then centrifuged again. The resulting supernatants were subjected to immunoprecipitation using either anti–NFI-A IgG or anti–NFI-C IgG. The immunoprecipitated materials were separated by SDS-PAGE, and the coprecipitated HA-tagged proteins were visualized by immunoblot analysis with anti-HA antibodies (Convance, Princeton, NJ).

RESULTS

Correlation between Induction of UGT1A2 and Appearance of CEREM-Binding Protein in Cultured Hepatocytes—Our previous studies indicated that CEREM was required for the transcriptional enhancement of UGT1A2 in primary hepatocytes, and that CEREM-binding proteins appeared in the nuclei of primary hepatocytes (*6*). Therefore, the induction of UGT1A2 is presumed to reflect increased CEREM-binding activity in the nuclei. To explore this possibility, we prepared nuclear extracts, total RNAs, and microsomal proteins from rat liver and cultured hepatocytes at various time points, and then analyzed the correlation of the culture-associated appearance of CEREM-binding activity with the time-dependent accumulation of UGT1A2 in primary hepatocytes.

We examined the culture-associated appearance of nuclear CEREM-binding activity during a specific period of cultivation. As shown in Fig. 1A, the gel mobility shift assay revealed the existence of a prominent DNA–protein complex in cultured hepatocytes. There was a marked increase in the intensity of CEREM-binding activity at 8 h after plating of hepatocytes (arrowhead). The intensities of the shift bands gradually increased up to 72 h after plating. In addition, there was a clear difference in the mobility of the shift bands between liver and cultured hepatocytes. A nuclear extract from liver gave much larger DNA–protein complexes, which were detected as multiple shift bands and exhibited slower mobilities than the major band detected for the cultured hepatocytes.

We next wanted to determine whether the activation of the UGT1A2 promoter was correlated with the appearance of nuclear CEREM-binding activity during a specific period of cultivation. We evaluated the expression of UGT1A2 mRNA by RT-PCR analysis of total RNA. A primer set for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the amounts of RNA. As shown in Fig. 1B, expression of UGT1A2 mRNA showed a rapid response on the cultivation of hepatocytes. The liver did not express a detectable level of UGT1A2 mRNA. A faint but significant amount of UGT1A2 mRNA was detectable 2 h after plating (data not shown), and a dramatic increase in UGT1A2 mRNA occurred after 8 h in culture. Comparison of the RT-PCR

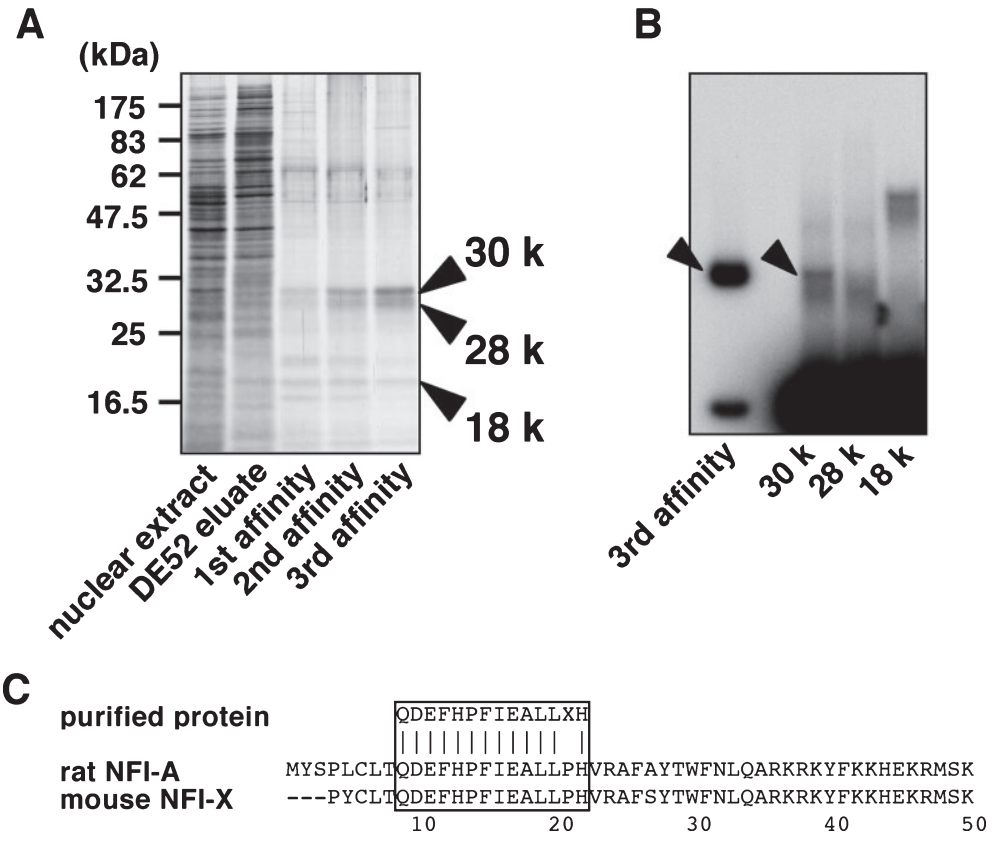


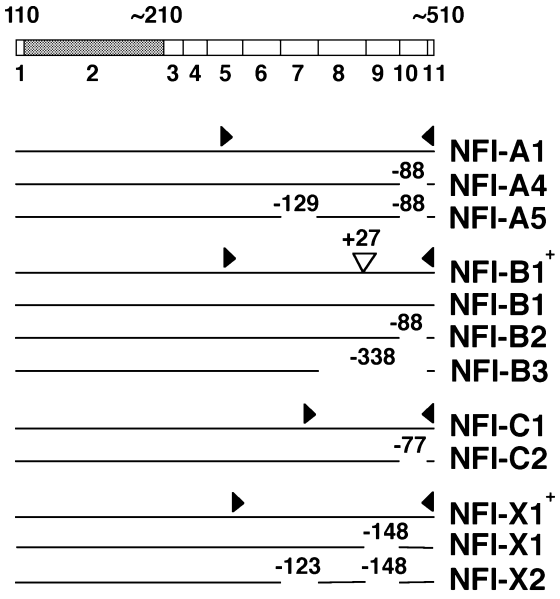
Fig. 2. **Purification and characterization of CEREM-binding proteins.** (A) SDS-PAGE analysis of CEREM-binding proteins at each purification step. Fractions were resolved on a 12% SDS–polyacrylamide gel, followed by visualization by silver staining. The positions of size markers are indicated on the left, and the three major polypeptide bands enriched after three passages through the CEREM-affinity column are indicated by arrowheads on the right. (B) Gel mobility shift assay with the renatured proteins. Affinity-purified CEREM-binding proteins ("3rd affinity" in panel A) were fractionated by SDS-PAGE. Three slices (containing the 30-, 28-, and 18-kDa protein bands) and one slice (a region between the 28-kDa and 18-kDa bands) were cut from the gel, and treated as described under "EXPERIMENTAL PROCEDURES." Samples were subjected to a gel mobility shift assay. The shifted bands are indicated by arrowheads. (C) Amino acid sequence of the NH_2-terminal of the purified 30-kDa polypeptide. The BLAST-P sequence similarity search results are shown below.

products showed that the maximum induction of UGT1A2 mRNA, which was about 80-fold higher than the value at time 0, was maintained at 48 h. Induction of UGT1A2 was observed but was somewhat reduced at 72 h. Furthermore, the elevated levels of UGT1A2 mRNA persisted for up to seven days after the plating of hepatocytes (data not shown). It is noteworthy that the UGT1A2 promoter was activated as early as 8 h after the initiation of the hepatocyte cultures.

Finally, to determine the amounts of UGT1A2 protein, microsomal proteins were examined by immunoblotting with a UGT1A2-specific antibody (*39*). This antibody gave a major band corresponding to an apparent molecular mass of 54,000 in cultured hepatocytes (Fig. 1C). COS cells, which had been transfected with an expression plasmid containing UGT1A2 cDNA, synthesized the same 54-kDa protein as that observed in the cultured hepatocytes (data not shown). Liver microsomes did not contain detectable amount of UGT1A2 protein. By contrast, the UGT1A2 protein level increased after the plating of hepatocytes. UGT1A2 was barely detectable 8 h after plating but became more evident 24 h after plating and continued to accumulate thereafter. On the other hand, expression of roughly equivalent amounts of UGT1A1 protein (52 kDa) was observed over the culture period. Taken together, these results demonstrate that CEREM-binding activity, which appears in the nuclei of primary cultures of hepatocytes, could be coupled to the culture-associated transcriptional enhancement of UGT1A2. These observations encouraged us to purify CEREM-binding proteins from the nuclei of cultured hepatocytes.

Purification and Characterization of CEREM-Binding Proteins from Primary Cultures of Rat Hepatocytes—We prepared nuclear extracts from 10 different primary hepatocyte preparations derived from 10 rats, and initially fractionated the pooled extracts by chromatography on a DE52 column. CEREM-binding activity was eluted at 0.2 M KCl from the DE52 column, and was further purified by DNA-affinity chromatography. CEREM-binding activity was eluted at 0.6 M NaCl from the affinity column. This eluted fraction was diluted to 0.1 M NaCl with five volumes of buffer A, and then subjected to further cycles through the same DNA-affinity column. Fractions at each purification step were examined by SDS-PAGE followed by silver staining (Fig. 2A). The eluate from the first DNA-affinity step gave many protein bands. After three passages through the DNA-affinity column, three major protein bands (30, 28, and 18 kDa) were enriched, the most abundant species being a 30-kDa protein.

To determine which protein band contained the CEREM-binding activity, we performed a denaturation–renaturation experiment (Fig. 2B). Three slices (containing the three major protein bands in Fig. 2A) and one slice (blank, representing a region between the 28 and 18 kDa bands) were cut from the SDS-PAGE gel, and then subjected to elution. The eluted samples were renatured and then examined by gel mobility shift assay. CEREM-binding activity was recovered from the gel slice contain-



length	amino acid	mol. wt
1,655 nt	509	55,975
1,567 nt	498	54,651
1,526 nt	455	51,482
1,556 nt	570	63,533
1,529 nt	561	62,717
1,441 nt	494	56,087
1,218 nt	420	47,498
1,506 nt	439	48,742
1,429 nt	428	47,536
>1,497 nt	n. d.	n. d.
1,349 nt	441	48,866
1,226 nt	400	44,651

(n. d. = not determined)

Fig. 3. **Schematic Illustration of NFI cDNA clones isolated from rat hepatocytes.** The general features of the four vertebrate NFI subfamilies are illustrated at the top, and schematic drawings of the 12 cDNA species are shown below. The numbers above the boxes are approximate amino acid residue numbers, and those below the boxes represent the 11 coding exons of the NFI genes. The hatched box depicts the highly conserved N-terminal 200 amino acids, spanning the DNA-binding and dimerization domain. The divergent C-terminal portion of the NFI protein is indicated by the open boxes. The locations of the primer pairs used in the RT-PCR analysis are indicated by closed arrowheads. The total residue numbers of the deduced amino acids and the calculated molecular weight of each isoform are listed on the right. Since we could not isolate a full-length NFI-X1$^+$ cDNA clone, the termination codon of NFI-X1$^+$ was not identified in this study.

ing the 30-kDa protein. To determine the NH_2-terminal amino acid sequences, the eluate from the third DNA-affinity step was separated by SDS-PAGE, followed by transfer to a PVDF membrane. The 30- and 28-kDa proteins were cut from the membrane and then subjected to automated protein sequencing. As shown in Fig. 2C, the 30-kDa protein did not have an NH_2-terminal initiator methionine, and its NH_2-terminal sequence was identical to the predicted sequence of the mammalian NFI family. However, the NH_2-terminal amino acid sequence of the 28-kDa protein could not be determined. Taken together, these results indicate that the 30-kDa protein, the major protein material in our most purified preparation, is active in CEREM-binding and is a member of the NFI family. This finding led us to isolate NFI cDNA from rat hepatocytes in order to determine which NFI family isoforms play a role in the transcriptional regulation of UGT1A2.

Isolation of cDNA Clones and Analysis of mRNA Expression of Alternative NFI Isoforms—The NFI family is encoded by four distinct but closely related genes in vertebrates (NFI-A, NFI-B, NFI-C, and NFI-X), each giving rise to multiple isoforms through alternative splicing of transcripts. We decided to obtain NFI family cDNA species by RT-PCR amplification using specific primer pairs for the four NFI genes, as described under "EXPERIMENTAL PROCEDURES." We identified 12 NFI cDNA species from rat hepatocytes: three NFI-A (A1, A4, and A5), four NFI-B (B1$^+$, B1, B2, and B3), two NFI-C (C1 and C2), and three NFI-X (X1$^+$, X1, and X2). Comparison of the structures of these 12 NFI cDNA species is schematically illustrated in Fig. 3. Analysis of the deduced amino acid sequences revealed that all of the NFI species isolated in this study had a highly conserved amino terminal portion (DNA-binding/dimerization domain) and a variable carboxy terminal portion (transcriptional activation/repression domain). The numbers of amino acids and the calculated molecular masses of the expected full-length proteins are also presented in Fig. 3. We could not obtain a full-length NFI-X1$^+$ cDNA clone which extended to the 3′-end, and could not identify its termination codon.

We determined the amount of mRNA encoding each NFI isoform by RT-PCR analysis of total RNA. Four sets of primer pairs were synthesized to amplify DNA fragments which span the spliced-out regions of each NFI subfamily (Fig. 3). As shown in Fig. 4, these primers generated PCR products of different length, and allowed us to detect and distinguish between the different NFI isoforms (splicing variants) isolated in this study. To evaluate the mRNA expression of each NFI isoform, the amount of RNA and the number of PCR cycles used were fixed at 1 μg and 20, respectively. The intensity of PCR product bands detected on autoradiography increased in proportion to the number of PCR cycles between 20 and 24 cycles (data not shown). Under these conditions, all assays were performed within the range of the linear relationship between the band intensity and the number of PCR cycles. A primer set for mouse GAPDH was used to normalize the amounts of RNA. NFI-A1 mRNA was the most abundant species in both liver and cultured hepatocytes. Small but significant amounts of mRNAs encoding NFI-C1 and NFI-X1 were also detected. It is noteworthy that the mRNA expression of these three isoforms did not show significant changes upon cultivation of hepatocytes. mRNA expression of the other NFI isoforms was barely detectable in liver and cultured hepatocytes.

Expression of NFI Family Proteins in Rat Liver and Cultured Hepatocytes—As described above, the diversity of the NFI protein family can be generated through alternative splicing of primary transcripts derived from the four individual NFI genes. Specific antibodies which discriminate between the four NFI families could be valuable tools for analyzing the expression of individual NFI

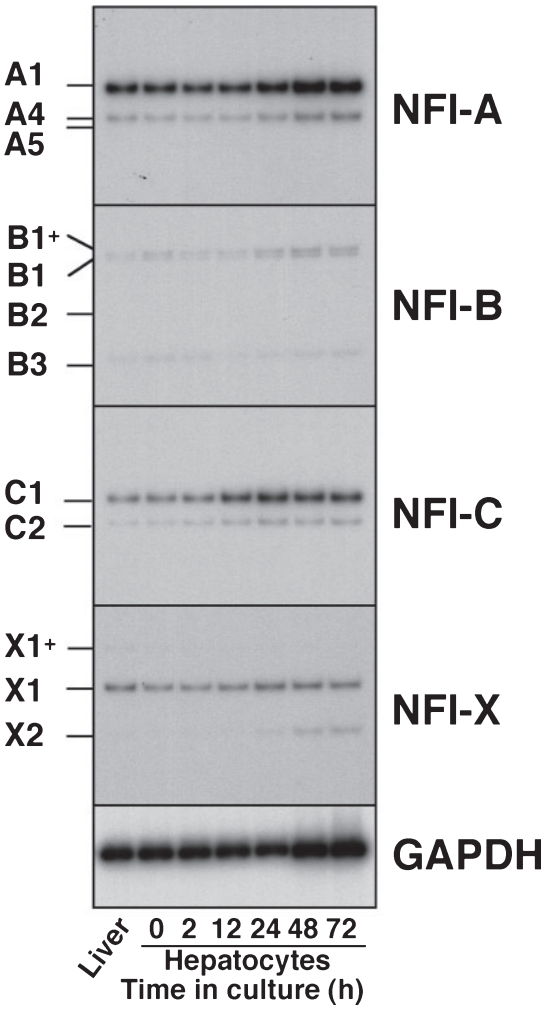


Fig. 4. **Expression profiles of NFI mRNAs on RT-PCR analysis.** Total RNA was prepared from rat liver and primary cultures of rat hepatocytes. Freshly isolated hepatocytes were plated at time zero and harvested at the indicated time points. Total RNA was treated with reverse transcriptase and then aliquots of the reaction mixtures were subjected to PCR using isoform-specific primer pairs. The ^{32}P-labeled PCR products were visualized by autoradiography. The predicted sizes of the products derived from the 12 NFI variants are shown on the left. Expression of GAPDH was analyzed for normalization of this assay, and serves as a control for equal amounts of RNA input.

family proteins. However, the commercially available antibodies recognize all the NFI isoforms, so we tried to prepare specific antibodies capable of distinguishing between the four NFI families. Amino acid residues 32–238 (NFI-A), 1–207 (NFI-B), 1–232 (NFI-C), and 140–400 (NFI-X) were individually expressed as fusion proteins with a 6× His tag in *E. coli*. These fusion proteins were separately purified by affinity chromatography on an Ni$^+$-NTA column. Anti–rat NFI antibodies were raised in male rabbits against the purified recombinant NFI proteins. As shown in Fig. 5A, Western blot analysis confirmed the immunoreactivities and the specificities of the four immunoglobulin (Ig) fractions against full-length NFI proteins expressed in COS cells. Anti–NFI-A Ig, anti–NFI-B Ig, and anti–NFI-X Ig were highly specific to NFI-A, NFI-B, and NFI-X, respectively. Anti–NFI-C Ig bound to NFI-C, and showed weak cross-reactivity with the other NFI family proteins. Thus, we were able to obtain antibodies capable of distinguishing between the four NFI family proteins. These antibodies should have wide application and facilitate the detailed analysis of NFI proteins.

Accumulating evidence has shown that NFI proteins can range in size from 40 to 100 kDa. This heterogeneity of NFI proteins arises from the alternative splicing of primary transcripts, and post-translational modifications such as phosphorylation and glycosylation (*16*, *24*). To identify the NFI species responsible for the culture-associated regulation of UGT1A2 expression, we analyzed the expression and time-dependent changes in NFI proteins in nuclear extracts by immunoblotting with specific antibodies (Fig 5B). When anti–NFI-A Ig was used for immunostaining, prominent immunoreactive protein bands were detected for rat liver and cultured hepatocytes. Anti–NFI-A Ig gave two major bands corresponding to apparent relative molecular masses of 63 and 61 kDa in rat liver. Expression of an extra protein with an approximate molecular mass of 58 kDa became evident 12 h after the plating of hepatocytes. The cellular contents of these NFI-A–related proteins seems to be maintained at nearly the same level during the first 48 h of cultivation, and more than 50% of the NFI-A–related proteins remained after 72 h cultivation. In contrast to anti–NFI-A Ig, anti–NFI-C Ig detected only one protein band in rat liver corresponding to an apparent molecular mass of 59 kDa. This 59-kDa protein disappeared immediately upon cultivation of hepatocytes. On the other hand, anti–NFI-B Ig and anti–NFI-X Ig did not give any prominent protein bands for either rat liver or cultured hepatocytes. These results suggest that one or more isoform within the NFI-A and NFI-C families, probably NFI-A1 and NFI-C1, might be involved in the culture-associated regulation of UGT1A2 expression.

Enhancement of CEREM-Mediated Expression of UGT1A2 by NFI-A1—To identify the NFI species responsible for the culture-associated regulation of UGT1A2 expression we performed a reporter assay, co-expressing a CEREM-containing reporter gene with each of the 12 NFI isoforms in cultured cells. To express NFI family proteins in mammalian cells, the NFI cDNAs which had been subcloned into pBluescriptII SK(+) were excised and recloned into the pUCDSRα vector. pA1-R/SRα has an NFI-A1 cDNA inserted in the reverse orientation and was used as a negative control. A reporter plasmid, CR66/pGL2-P, in which the 66-bp CEREM sequence was inserted immediately upstream of the heterologous SV40 promoter, was transfected into HepG2 cells in the presence or absence of NFI expression plasmids. Luciferase activity was measured in the cell extracts, normalized as to β-galactosidase activity, and expressed as a ratio to the luciferase activity on co-transfection with pA1-R/SRα. As shown in Fig. 6, luciferase activity increased upon co-transfection of the reporter gene with any one of NFI-A1, NFI-A5, NFI-B1$^+$, NFI-B1, NFI-B2, NFI-B3, and NFI-X1$^+$. By contrast, NFI-C1 and NFI-C2 tended to suppress the CEREM-mediated expression of the reporter gene in transfected cells, whereas NFI-A4, NFI-X1, and NFI-X2 had little direct effect on luciferase activity. Taken

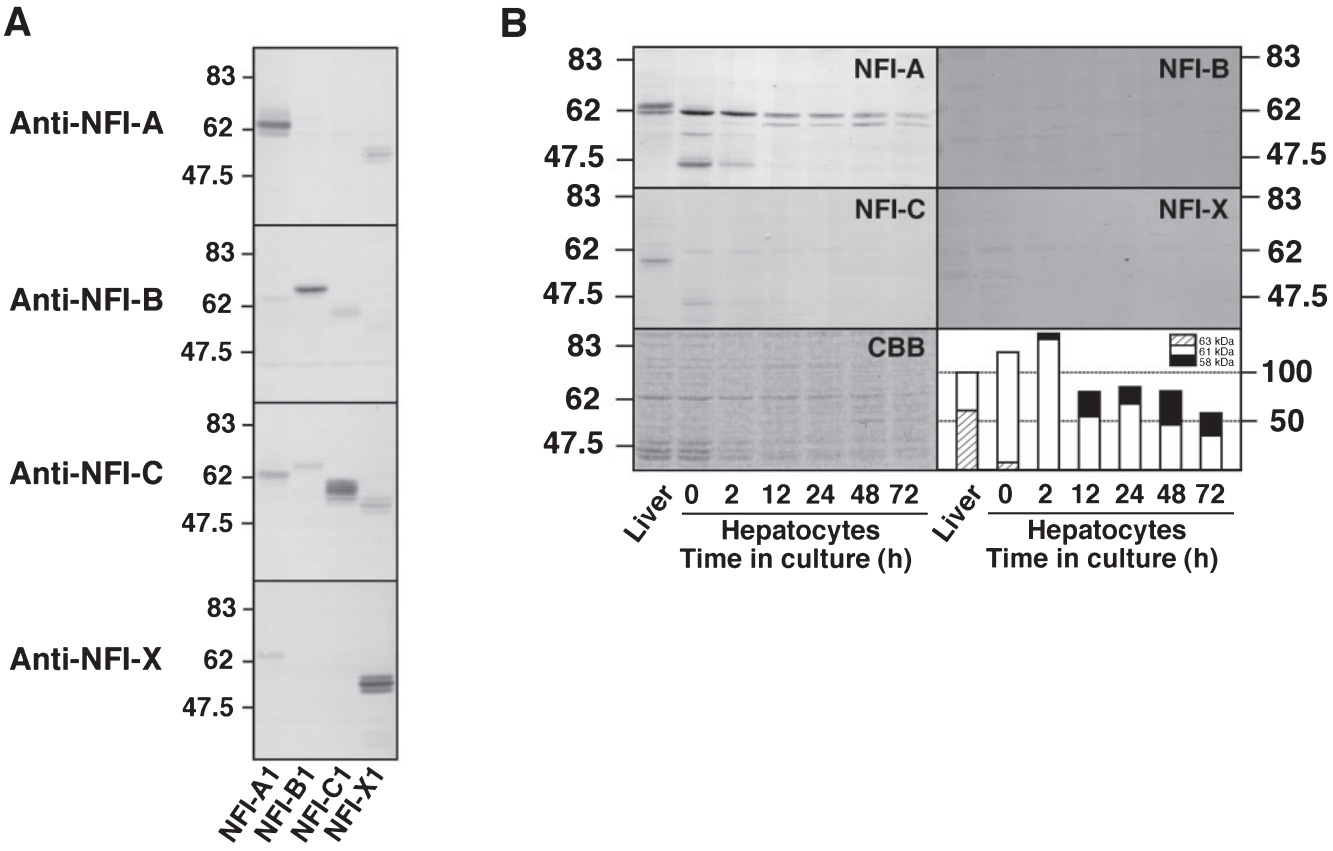


Fig. 5. **Time-dependent changes in nuclear NFI proteins in primary cultures of rat hepatocytes.** (A) Confirmation of antibody specificity by immunoblot analysis. Four different antibodies were raised against affinity purified recombinant proteins fused with a 6× His tag as described under "EXPERIMENTAL PROCEDURES." Their immunoreactivities and specificities were confirmed by immunoblot analysis using the full-length NFI-A1, NFI-B1, NFI-C1, and NFI-X1 proteins expressed in COS cells in the presence of the antiserum at 1:5,000 dilution. The positions of standard molecular weight markers are indicated on the left of each panel. (B) Time-dependent changes in NFI proteins in primary cultures of rat hepatocytes. Freshly isolated hepatocytes were plated at time zero, and harvested at 0, 2, 12, 24, 48, and 72 h after plating. Nuclear extracts were prepared from rat liver and cultured hepatocytes. Ten micrograms of each nuclear extract was subjected to immunoblot analysis using the four different antibodies. The same region of the gel shown in the immunoblot analysis was stained with Coomassie Brilliant Blue and analyzed as a loading control. Quantitative data for the three NFI-A–related protein bands (63, 61, and 58 kDa) were determined with a LAS1000 image analyzer, expressed relative to those of CBB-stained protein bands, and summarized as percentages of the total NFI-A protein. The positions of standard molecular weight markers are indicated on the left of each panel.

together, the results presented in Figs. 5 and 6 suggest that NFI-A1 and NFI-C1 might play key roles in the culture-associated regulation of UGT1A2 expression.

It was also demonstrated that NFI-A1 and NFI-C1 have opposite effects on the regulation of UGT1A2, that is, up-regulation by NFI-A1 and down-regulation by NFI-C1. Under these circumstances, the disappearance of NFI-C1 in cultured hepatocytes might trigger the culture-associated transcriptional enhancement of UGT1A2 mainly under the control of NFI-A1. To explore this possibility, we examined whether co-expression of NFI-A1 with NFI-C1 could influence the CEREM-mediated expression of the reporter gene in a dose-dependent manner. CR66/pGL2-P was co-transfected with constant amounts of pA1/SRα (NFI-A1) and increasing amounts of pC1/SRα (NFI-C1) into HepG2 cells. As shown in Fig. 7, NFI-A1 (lane 2) increased CR66/pGL2-P activity approximately 3-fold compared to pA1-R/SRα (lane 1), while NFI-C1 (lane 7) decreased it to less than 80%. Co-expression of NFI-A1 with NFI-C1 led to a concentration-dependent decrease in the transcriptional enhancement mediated by NFI-A1 of the CR66/pGL2-P reporter gene. Co-transfection of equal amounts of pA1/SRα and pC1/SRα (lane 6) resulted in a 70% loss of NFI-A1-mediated activation of CR66/pGL2-P expression (compare with lane 2).

It was postulated that the number and the mode of binding of NFI-A and NFI-C proteins which interact with CEREM might influence the expression of UGT1A2. In fact, the gel mobility shift assay in Fig. 1A showed that DNA–protein complexes were detected as multiple shift bands, and that there was a clear difference in the mobility of the shift bands between liver and cultured hepatocytes. Therefore, we wanted to determine whether these different DNA–protein complexes, especially the larger ones seen in the nuclear extract of liver, contain distinct kinds of NFI proteins. To explore this possibility, the nuclear proteins were separated on a native gel in the presence or absence of DNA probe, where only DNA–protein complexes could enter the native gel. The gel was electroblotted onto a nitrocellulose membrane, the DNA

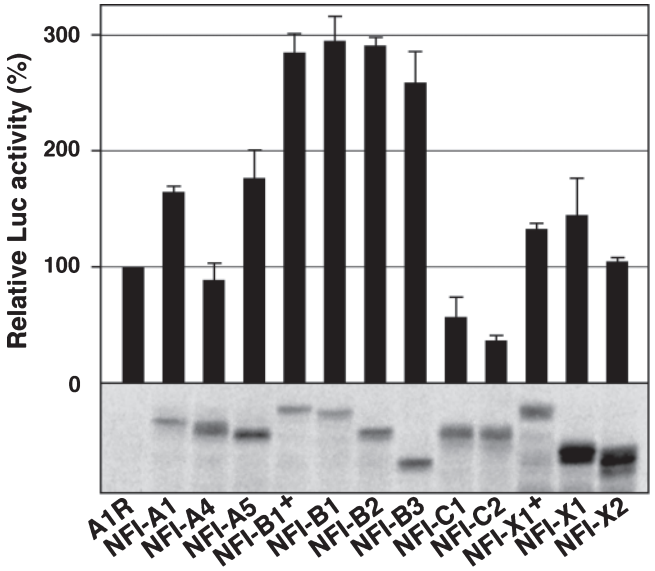


Fig. 6. **Effects of the 12 NFI isoforms on expression of the reporter gene controlled by CEREM.** A luciferase reporter gene (CR66/pGL2-P) bears the 66-bp CEREM sequence immediately upstream of the SV40 promoter. This reporter plasmid (0.9 μg) was transiently transfected into HepG2 cells in the presence of expression plasmids (0.9 μg) encoding distinct isoforms of the NFI family and pmiwZ (0.2 μg) encoding β-galactosidase. pA1-R/SRα had an NFI-A1 cDNA inserted in the reverse orientation in the pUCDSRα vector, and was used as a negative control for the expression of NFI proteins. Cells were harvested at 40 h post-transfection. Luciferase activity was measured in the cell extracts, normalized as to β-galactoidase activity, and expressed as the ratio to the luciferase activity on co-transfection with pA1-R/SRα. All values are expressed as means ± SD of three independent experiments. Expression of the transfected cDNAs was also confirmed by immunoblot analysis with anti-NFI (N20) IgG. The blot presented here is representative of three independent experiments.

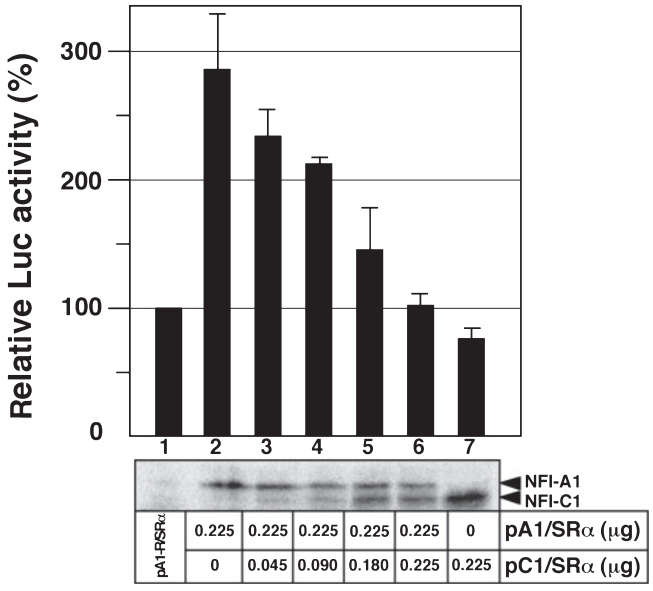


Fig. 7. **Activation of CEREM-mediated expression of the reporter gene by NFI-A.** Luciferase reporter plasmid CR66/pGL2-P (0.45 μg) was transiently transfected into HepG2 cells in the presence of a DNA mixture of expression plasmids (0.45 μg) and pmiwZ (0.1 μg). The 0.45-μg DNA mixture consisted of the indicated amounts of pA1/SRα and pC1/SRα, and empty vector pUCDSRα was supplied to adjust the total amount of plasmid DNA to 0.45 μg. pA1-R/SRα was used as a negative control for the expression of NFI proteins. Cells were harvested at 40 h post-transfection. Luciferase activity was measured in the cell extracts, normalized as to β-galactosidase activity, and expressed as the ratio to the luciferase activity on co-transfection with pA1-R/SRα. All values are expressed as means ± SD of three independent experiments. Expression of the NFI-A and NFI-C proteins was also confirmed by immunoblot analysis with anti-NFI (N20) IgG.

probe was visualized by autoradiography, and then the proteins were visualized by immunostaining with anti–NFI-A Ig or anti–NFI-C Ig. As shown in Fig. 8A, the immunoreactive proteins did not enter the native gel without the DNA probe, while they moved into the gel as complexes with specific DNA. NFI-A proteins formed larger DNA–protein complexes in the nuclear extract from liver (closed arrowhead #1), although they formed smaller complexes in the nuclear extract from cultured hepatocytes (open arrowhead). NFI-C proteins also formed larger DNA-protein complexes in the liver nuclear extract (closed arrowhead #2), but disappeared in the cultured hepatocytes. We could not observe direct interaction between NFI-A proteins and NFI-C proteins on the same DNA molecule containing CEREM under these *in vitro* assay conditions. NFI-B and NFI-X proteins were not detected in this assay (data not shown). The faint bands indicated by asterisks in lanes "plus anti–NFI-C IgG" were probably produced through possible cross-immunoreaction against NFI-A proteins. As shown in Fig. 8B, supershift assays performed with antibodies against NFI-A and NFI-C indicated that only anti–NFI-A gave a slower migrating complex. Under these assay conditions, the major CEREM-binding complex seen in cultured hepatocyte nuclear extracts appeared to contain NFI-A.

To further study the association of NFI-A1 and NFI-C1, we then examined this possibility by immunoprecipitation (Fig. 8C). NFI-C1 was immunoprecipitated from HepG2 cells which express NFI-C1 and HA-tagged NFI-A1, and examined for the presence of HA-tagged NFI-A1. Western blot analysis clearly showed that HA-tagged NFI-A1 was coprecipitated with NFI-C1 only when NFI-C1 was coexpressed (Fig. 8C, left panel). Likewise, coexpression of NFI-A1 and HA-tagged NFI-C1 resulted in coprecipitation of HA-tagged NFI-C1 with NFI-A1 (Fig. 8C, right panel). These results demonstrate that direct interaction between NFI-A1 and NFI-C1 takes place in the cells.

DISCUSSION

UGT1A2 gene regulation has some unique features that contrast with those of the other UGT1 isoforms. Expression of UGT1A2 is very low in the rat liver, while it is highly induced in hepatocyte primary cultures. The 66-bp *cis*-acting enhancer module, CEREM, is located between –307 and –242 upstream of the transcription start site and is required for the culture-associated induction of UGT1A2 in primary hepatocytes (*6*). In this report, we demonstrate a potential role for NFI proteins, especially NFI-A1 and NFI-C1, in the culture-associated transcriptional regulation of the rat UGT1A2 gene. We also dem-

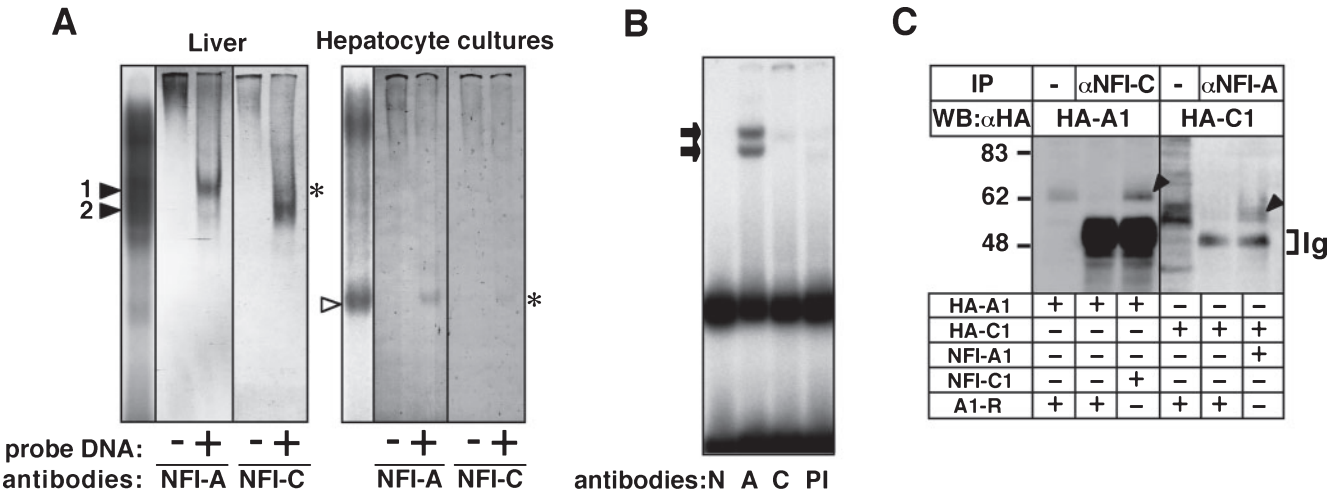


Fig. 8. **Interaction between NFI-A1 and NFI-C1.** (A) Immunostaining of the shifted NFI-A and NFI-C proteins. Crude nuclear extracts were prepared from rat liver and primary rat hepatocytes that had been cultured for 72 h. Gel shift reaction mixtures with or without 66-base pair DNA probe –307/–242 were fractionated by native PAGE. The gel was electroblotted onto a membrane. The DNA was revealed by autoradiography, and the proteins were revealed by immunostaining with either anti–NFI-A Ig or anti–NFI-C Ig. Possible cross-reaction of anti–NFI-C IgG with NFI-A proteins is indicated by asterisks. (B) Supershifts demonstrating binding of NFI-A to CEREM. Crude nuclear extracts from rat primary hepatocytes were preincubated with the indicated antibodies (2 μg) before being incubated with the ^{32}P-labeled DNA probe. The arrow indicates the band shifted by anti–NFI-A. The lanes are indicated as follows: N, untreated; A, anti–NFI-A IgG; C, anti–NFI-C IgG; PI, preimmune IgG. (C) Co-immunoprecipitation of the NFI-A and NFI-C proteins. HepG2 cells were transfected with the indicated combinations of plasmids. Cells were harvested at 40 h post-transfection, and then subjected to immuoprecipitation (IP) using either anti–NFI-C IgG (left panel) or anti–NFI-A IgG (right panel). The resulting antigen–antibody complex was recovered by use of protein A–Sepharose. The immunoprecipitates were analyzed by Western blotting (WB) with monoclonal antibodies against HA.

onstrate that the composition of NFI proteins in the nuclei shows a dramatic change in association with the cultivation of hepatocytes, which raises the possibility that this alteration might trigger the culture-associated expression of UGT1A2.

First, we examined in detail the correlation between the expression of UGT1A2 and the appearance of nuclear CEREM-binding activity in primary hepatocytes. Culture-associated expression of UGT1A2 mRNA shows a very rapid response on the initiation of cultivation of isolated hepatocytes. Accumulation of UGT1A2 mRNA was evident at 2 h after plating of hepatocytes, and a dramatic increase in UGT1A2 mRNA had occurred by 8 h culture. Gel mobility shift assays showed the nuclear CEREM-binding activity as multiple shift bands. Liver contained larger DNA–protein complexes which rapidly disappeared in cultured hepatocytes. Instead, cultured hepatocytes contained smaller DNA-protein complexes. The appearance of the smaller species in the nuclei of cultured hepatocytes was closely correlated with the initiation of the culture-associated induction of UGT1A2 mRNA. Another line of evidence supports this finding: a base substitution in the core binding motif of CEREM, which alters 5′-TGGCAC-3′ to 5′-TTTAAC-3′ (between –273 and –268), reduced culture-associated expression of the reporter gene to the basal level and decreased the formation of a DNA-protein complex observed in the gel mobility shift assay (Y. Emi, unpublished results). This result further emphasizes the close correlation between inhibition of the protein-binding to CEREM and the reduction in the CEREM-mediated expression of the reporter gene. In addition, it is noteworthy that the rapid disappearance of the larger species upon cultivation of hepatocytes seemed to be involved in the early phase of the culture-associated induction of UGT1A2. The molecular details of the larger complex are the subject of our current research.

Second, we purified and characterized specific CEREM-binding proteins from the nuclei of cultured hepatocytes. The purified proteins gave at least three major protein bands (30, 28, and 18 kDa), the most abundant species being a 30-kDa protein. The NH_2-terminal sequence of the 30-kDa protein revealed it to be a member of the NFI family. NFI proteins consist of various isoforms ranging in size from 40 to 100 kDa. The diversity of the NFI protein family is generated by four distinct but closely related genes, NFI-A, NFI-B, NFI-C, and NFI-X, through differential splicing and post-translational modifications such as limited proteolysis, glycosylation, and phosphorylation (*16*, *24*). Heterodimer formation between individual members of a protein family can provide an additional mechanism of diversity increasing the number of DNA-binding proteins (*40*). Thus, the multiple bands observed in the gel mobility shift assay are probably due to these properties of the NFI family members. Paonessa et al. described the purification of a NFI-related protein from rat liver and referred to it as NFI-L (*18*). NFI-L binds to the TGGCA-motif in the human albumin promoter and has an apparent molecular mass of 30 kDa. The NH_2-terminal sequence of NFI-L is nearly identical to that of CEREM-binding protein. Since the core sequence of CEREM has the same TGGCA-motif, it is not surprising that we purified a similar 30-kDa protein from primary cultures of rat hepatocytes. In the light of our results obtained on the isolation of NFI cDNA clones and immunoblot analysis of NFI proteins, we con-

sider that the purified 30-kDa CEREM-binding protein is a cleavage derivative of larger NFI molecules that were produced during the processes of extraction and purification. Since it is well known that NFI proteins contain a highly conserved NH_2-terminal DNA-binding and dimerization domain (approximately 200 residues), this 30-kDa species still retained its DNA-binding activity during the purification steps.

Third, we isolated 12 cDNA species encoding different NFI isoforms from rat hepatocytes, and carried out reporter assays involving co-transfection of a reporter gene with one of the 12 NFI isoforms into HepG2 cells. It can be generalized that CEREM-controlled expression of UGT1A2 is up-regulated by NFI-A and NFI-B, down-regulated by NFI-C, and not affected by NFI-X. We analyzed the mRNA expression profile of each NFI isoform by RT-PCR. NFI-A1 was the most abundant mRNA species in both liver and cultured hepatocytes, NFI-C1 and NFI-X1 being also expressed as additional mRNA species. Immunoblot analysis of nuclear extracts showed that NFI-A–related polypeptides were the major components of NFI family proteins in both liver and cultured hepatocytes. By contrast, NFI-C–related polypeptides were only present in liver nuclei, and disappeared immediately on the cultivation of hepatocytes. In addition, NFI-B and NFI-X proteins were not detectable in liver and cultured hepatocytes. These results demonstrate that NFI-A1 and NFI-C1 have opposite effects on the CEREM-mediated regulation of UGT1A2, and play key roles in the culture-associated expression of UGT1A2. Indeed, in the reporter assay with HepG2 cells, co-expression of NFI-A1 with increasing amounts of NFI-C1 led to a concentration-dependent decrease in the transcriptional activation of the reporter gene which was up-regulated by NFI-A1. Co-immunoprecipitation experiments also demonstrated the association of NFI-A1 and NFI-C1. Thus, the co-existence of NFI-A1 and NFI-C1 in liver nuclei results in the formation of the NFI-A1 homodimer, NFI-A1/NFI-C1 heterodimer, and NFI-C1 homodimer. We conclude that the latter two forms account for suppression of the CEREM-controlled expression of UGT1A2 to the basal level, and that the rapid disappearance of NFI-C1 in cultured hepatocytes trggers the culture-associated transcriptional enhancement of UGT1A2 that is mainly under the control of NFI-A1.

Since there is such diversity in the NFI family generated through various modifications such as alternative splicing of primary transcripts, the analysis of NFI protein expression has been shown to be extremely complicated and controversial. To solve this problem, we attempted to prepare specific antibodies which could distinguish between the four NFI families. In this report, by use of such specific antibodies, we determined the detailed expression profiles of the four NFI family proteins in rat liver and cultured hepatocytes, and clearly demonstrated the culture-associated alteration of NFI protein expression. NFI-A1 was found to be a major component of the NFI family in liver and cultured hepatocytes. Liver nuclei contained 63- and 61-kDa species. The 61-kDa species might have resulted from proteolytic cleavage of the 63-kDa species, since immunoblot analysis of COS cells, which express NFI-A1, gave the same two protein bands. On the other hand, cultured hepatocytes contained 61-kDa and 58-kDa species. These two smaller protein species are also thought to be proteolytic derivatives of NFI-A1 which had been truncated by 10–20 residues on each side of the polypeptide. An unexpected finding in this study was that the amounts of nuclear NFI-C and NFI-X proteins did not coincide with the cellular amounts of their mRNAs. NFI-X1 mRNA was detected in liver and cultured hepatocytes in similar amounts, but detectable amounts of NFI-X proteins were not found in liver and cultured hepatocytes. Interestingly, similar amounts of NFI-C1 mRNA were detected in liver and cultured hepatocytes, but NFI-C proteins disappeared immediately upon cultivation of isolated hepatocytes. The ablation of NFI-C from cultured hepatocytes has important implications for the culture-associated transcriptional enhancement of UGT1A2. Although the molecular mechanisms underlying these discrepancies are unknown, these phenomena are thought to be caused by post-translational modifications, such as the accelerated degradation of newly synthesized polypeptides. Proteolysis-assisted control of the ability of NFIs to regulate their target genes, such as UGT1A2, has become the subject of considerable interest. Gao et al. reported that the level of the NFI protein is downregulated in the regenerating rat liver (*41*). An analogous decrease in NFI-C1 is also observed in hepatocyte primary cultures. Further work is needed to determine the changes in the half-lives of NFI proteins upon cultivation of hepatocytes, and to identify the protease(s) involved in the degradation of NFI-C proteins.

Recently, NFI was implicated in the regulation of genes encoding drug-metabolizing enzymes such as glutathione *S*-transferase (GST) and cytochrome P-450 (CYP), and it was also proposed to be a possible modulating factor for the drug-responsiveness, tissue-specificity, and redox regulation of these genes. Expression of GST-P is completely repressed in rat liver, and NFI-A family proteins have been shown to be involved in silencing of this gene in the liver (*34*). Olfactory mucosa–specific expression of the CYP1A2 and CYP2A3 genes was found to be controlled by NFI (*42*, *43*). In addition, it has also been found that NFI family proteins can mediate cross-talk with other regulatory factors. An NFI binding site was found in the dioxin-responsive mouse *Cyp1a1* gene. In mouse hepatoma cells it was shown that the binding of the ligand-activated dioxin receptor complex to its recognition site in the *Cyp1a1* gene facilitates the binding of NFI to its site next to the TATA-box of this gene (*44*). Of particular interest is that NFI recognition sites were also found in the phenobarbital-responsive enhancer unit (PBRU) of the rat *CYP2B2* and mouse *Cyp2b10* genes, and are flanked by two potential nuclear receptor binding sites, NR1 and NR2 (*45*, *46*). A heterodimer of the constitutive androstane receptor (CAR) and the retinoid X receptor (RXR) bound to the core NR-1 and NR-2 sites, and the CAR/RXR-mediated trans-activation of the PBRU was increased in the presence of NFI. CAR/RXR and NFI bind independently and simultaneously to the PBRU, and perhaps mediate the co-operative recruitment of co-activators to the PBRU (*47*).

In the case of UGT1A2, our previous DNase I protection analysis revealed the existence of a half-site of the NFI-binding motif, 5′-TGGCAC-3′, in CEREM (*6*).

CEREM contains two additional protein-binding sites, one is a characteristic repetitive sequence immediately upstream of the NFI-binding site and the other a consensus sequence for activator protein-1 downstream of the NFI site. Though protein binding to the two potential sites was not demonstrated in this study, a NUBIScan (*48*) predicted a plausible nuclear receptor binding site (DR-1) in the upstream repetitive sequence. In fact, methylation of guanine residues within this motif interfered with the CEREM-binding activity (*6*). These findings imply that an additional factor, probably a member of the nuclear receptor family, might interact with a distinct region in CEREM and might cooperate with the NFI protein. Liver contains larger DNA–protein complexes while cultured hepatocytes contain smaller DNA–protein complexes. This dramatic size difference cannot be explained entirely by the culture-associated ablation of NFI-C1 and the truncation of NFI-A1, and is likely to be accounted for by the dissociation of the NFI dimer and the departure of other regulatory cofactor(s), including the nuclear receptor family. Further research is ongoing to clarify these points.

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