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Expression Patterns of the Four Nuclear Factor I Genes During Mouse Embryogenesis Indicate a Potential Role in Development

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ABSTRACT **The nuclear factor I (NFI) family of site-specific DNA-binding proteins is required for both the cell-type specific transcription of many viral and cellular genes and for the replication of adenovirus DNA. Although binding sites for NFI proteins within the promoters of several tissue-specific genes have been shown to be essential for their expression, it is unclear which NFI gene products function in specific tissues during development. We have isolated cDNAs from all four murine NFI genes (gene designations *Nfia, Nfib, Nfic, and Nfix*), assessed the embryonic and postnatal expression patterns of the NFI genes, and determined the ability of specific NFI proteins to activate transcription from the NFI-dependent mouse mammary tumor virus (MMTV) promoter. In adult mice, all four NFI genes are most highly expressed in lung, liver, heart, and other tissues but only weakly expressed in spleen and testis. The embryonic expression patterns of the NFI genes is complex, with NFI-A transcripts appearing earliest—within 9 days postcoitum in the heart and developing brain. The four genes exhibit unique but overlapping patterns of expression during embryonic development, with high level expression of NFI-A, NFI-B, and NFI-X transcripts in neocortex and extensive expression of the four genes in muscle, connective tissue, liver, and other organ systems. The four NFI gene products studied differ in their ability to activate expression of the NFI-dependent MMTV promoter, with the NFI-B protein being most active and the NFI-A protein being least active. These data are discussed in the context of the developmental expression patterns of known NFI-responsive genes. The differential activation of an NFI-dependent promoter, together with the expression patterns observed for the four genes, indicate that the NFI proteins may play an important role in regulating tissue-specific gene expression during mammalian embryogenesis. *Dev. Dyn. 208:313–325, 1997.***

Key words: development; mouse embryo; nuclear factor I; transcription

INTRODUCTION

The differential expression and function of individual members of multigene families of transcription factors are essential for proper development. For example, the correct spatial and temporal expression of specific members of the Hox, HLH, Pax, and GATA multigene families of transcription factors play integral roles in mouse embryogenesis (see Krumlauf, 1994; Olson and Klein, 1994; Stuart et al., 1994; Simon, 1995 for reviews). As an initial step in the analysis of the role of the nuclear factor I (NFI) family of transcription/replication factors in mammalian development, we have assessed the developmental expression patterns and transcriptional activation properties of the four members of the NFI gene family.

NFI was originally identified as a host-encoded protein required for efficient initiation of adenovirus replication in vitro (Nagata et al., 1982) and was later shown to function in the expression of a number of cellular genes. Cloning of cDNAs encoding NFI proteins from a number of species (Paonessa et al., 1988; Santoro et al., 1988; Meisterernst et al., 1989; Rupp et al., 1990) has identified a family of four genes (NFI-A, NFI-B, NFI-C, and NFI-X) that are highly conserved from chicken to human. NFI proteins share a highly conserved N-terminal 220 amino acid domain, which mediates DNA binding, dimerization, and the initiation of adenovirus replication (Mermod et al., 1989; Gounari et al., 1990; Bandyopadhyay and Gronostajski, 1994). NFI proteins bind to DNA as both homodimers and heterodimers, all of which appear to recognize the consensus binding site, TTGGC(N5)GCCAA, with the same apparent affinity (Goyal et al., 1990; Kruse and Sippel, 1994b). However, considerable variation occurs within the C-terminal domains of the NFI proteins, which likely encode transcription modulation domains. Additional variation between NFI proteins is generated through differential splicing of transcripts from each of the four genes (Kruse and Sippel, 1994a).

The precise mechanism of NFI-mediated transcriptional modulation is not known; however, interactions

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between the C-terminal proline/serine-rich domain of a human NFI-C isoform (CTF1) and components of the basal transcriptional machinery have been detected (Altmann et al., 1994; Kim and Roeder, 1994; Xiao et al., 1994). These findings suggest that the C-terminal transactivation domain of NFI-C may function by enhancing recruitment of components of the transcription apparatus to promoters containing NFI binding sites, thereby increasing the rate of transcription. Whether the other NFI proteins function in a similar manner is unknown.

Binding sites for NFI proteins are present in cellular genes expressed in multiple tissues (Quinn et al., 1988) and in genes expressed solely in brain (Aoyama et al., 1990; Amemiya et al., 1992), muscle (Darville et al., 1992; Edmondson et al., 1992), liver (Cereghini et al., 1987), mammary gland (Li and Rosen, 1995), and other differentiated cell types (Graves et al., 1991). Mutational analysis indicates these sites are required for the proper expression of many tissue-specific and developmentally regulated genes (Knezetic and Felsenfeld, 1993). NFI proteins have also been shown to suppress transformation of cells by nuclear oncogenes. Overexpression of avian NFI proteins in chick embryo fibroblasts (CEF) reduces focus formation by the *jun, fos, junD, myc,* and *qin* oncogenes (Schuur et al., 1995). The mechanism of this suppression is unknown; however, overexpression of NFI induces several morphologic changes including increased cell adherence and flattening the cell monolayer, which may be required for the suppression.

To date, it has been difficult to define which NFI gene products function in vivo at specific promoters because the expression pattern of the NFI multigene family is poorly understood. We have recently shown that the four NFI genes are differentially regulated during phorbol ester (TPA)-induced differentiation of human leukemic cells and may play an important role in hematopoietic development (Kulkarni and Gronostajski, 1996). Here we describe the cloning of cDNAs of the murine *Nfia, Nfib, Nfic,* and *Nfix* genes, analysis of the expression patterns of the NFI genes during mouse development, and functional characterization of the transactivation properties of specific NFI isoforms. In contrast to earlier studies that suggested that NFI proteins were constitutively expressed in mammalian cells, we demonstrate here that the four NFI genes are expressed in distinct patterns during mouse development and that the NFI gene products differ in their transcriptional activation of an NFI-dependent promoter.

RESULTS

Cloning of cDNAs From the Four Murine NFI Genes and Analysis of Their Expression in Adult Tissues

Although cDNAs encoding NFI proteins from a number of species have been cloned previously, there has been no detailed description of the tissue-specific expression of this multigene family of transcription/replication proteins or comparative analysis of their transcription activation properties. To study the developmental expression and biochemical properties of murine NFI proteins, we cloned cDNAs representing each of the four murine NFI genes. Degenerate oligonucleotides were used to amplify mouse genomic DNA, and individual polymerase chain reaction (PCR) products were cloned and sequenced. Sequences of the cloned PCR products showed proteins homologous to the four chicken and human NFI proteins (Fig. 1). Because PCR products of the same size were generated from each of the four murine NFI genes (486 bp, data not shown), these data indicate that the primers were contained within single exons of all four mouse NFI genes, as was seen previously for the porcine NFI-C gene (Meisterernst et al., 1989) and the four human NFI genes (Kulkarni and Gronostajski, 1996). Oligonucleotide probes within these PCR products (Fig. 1) were used to screen mouse liver and skeletal muscle cDNA libraries to obtain full-length cDNAs. The predicted protein products from the four murine cDNAs are homologous to the previously described chicken NFI-A1.1, NFI-B2, NFI-C2, and human NFI-X2 proteins, for which the numbers following the gene designation indicate conserved splicing patterns seen in avian and mammalian NFI cDNAs (Kruse and Sippel, 1994a). The four cDNAs share features seen in other cloned NFI cDNAs, including the presence of a highly homologous DNA-binding/dimerization domain located from residues 1–245 of each protein and C-terminal domains that are gene-specific and conserved from chickens to humans.

To assess the expression pattern of the four murine NFI genes in adult tissues, gene-specific probes (Fig. 1) were used to analyze multitissue polyA$^+$ Northern blots (Fig. 2). The levels of NFI transcripts were normalized to the level of S26 ribosomal protein transcripts detected in reprobing of the same blots (data not shown). The NFI-A probe detected major transcripts of 10.5 kb and 5 kb, similar in size to those seen previously using a 3′ region of the mouse NFI-A gene (Inoue et al., 1990). NFI-A transcripts were most abundant in heart, lung, and kidney, with lower levels of expression in liver, skeletal muscle, spleen, brain, and testis. The NFI-B probe detected a transcript of 9.7 kb with maximum expression in lung, skeletal muscle, and heart; lower level expression in liver, kidney, and brain; and very low level expression in testis and spleen. The NFI-C probe detected transcripts of 7.7 kb and 4.2 kb, similar to the sizes seen previously in mouse and human tissues using 3′ regions of the human NFI-C cDNA as a probe (Santoro et al., 1988). The highest level of expression of NFI-C transcripts was in skeletal muscle, with intermediate levels in heart, liver, kidney, lung, and brain and much lower levels in testis and spleen. The NFI-X probe detected a major transcript of 6 kb, with an expression pattern similar to that of the NFI-C probe with maximal expression in skeletal muscle; intermediate expression in heart, liver, kidney, lung, and brain; and minimal expression in testis and spleen. Thus, testis and

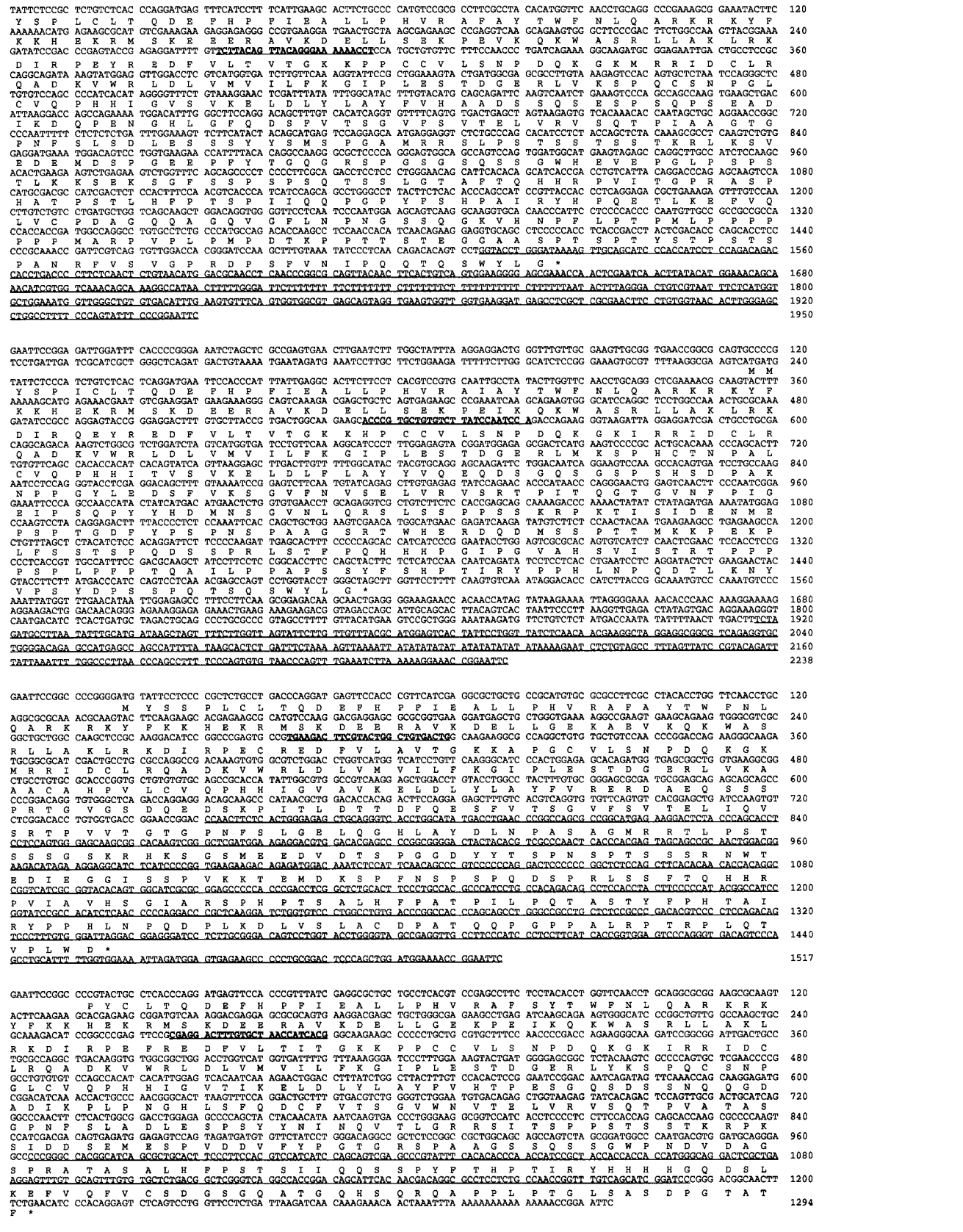


Fig. 1. Nucleotide sequences and theoretical translations of NFI-A, NFI-B, NFI-C, and NFI-X cDNAs. The sequences of the predicted polypeptides are shown below each nucleic acid sequence. The sequences complementary to the oligonucleotides used for Northern analysis and isolation of cDNAs are in bold and underlined print. Sequences complementary to cRNA probes used for in situ analyses are underlined. The PCR fragments generated from mouse genomic DNA using the Deg1 and Deg2 primers are equivalent to residues 51–478 of mNFI-A, 291–718 of mNFI-B, 74–501 of mNFI-C, and 56–483 of mNFI-X. Genbank accession numbers are as follows: mNFI-A, U57633; mNFI-B, U57634; mNFI-C, U57635; and mNFI-X, U57636.

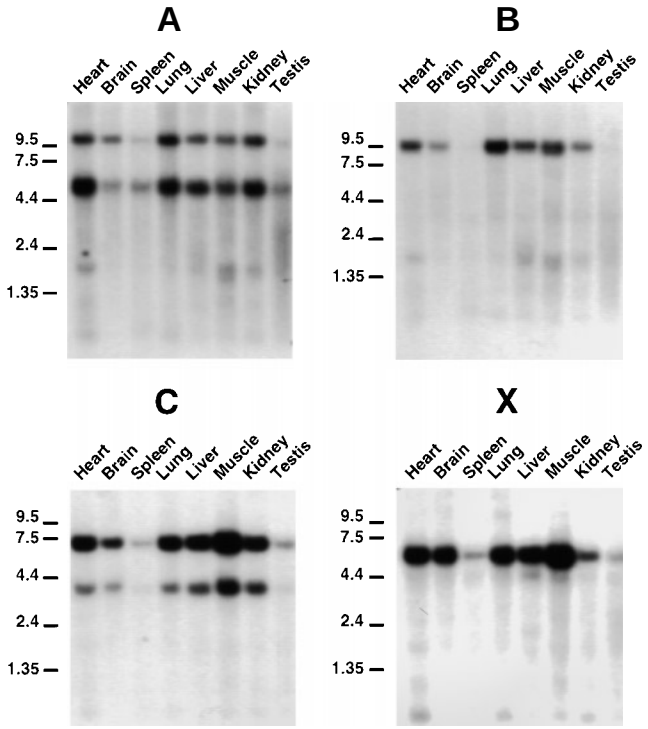


Fig. 2. Tissue-specific expression of NFI mRNAs in adult mice. Oligonucleotides specific for each of the four murine NFI genes (Fig. 1) were hybridized to multiple tissue Northern blots containing 2 μg polyA$^+$ RNA (Clontech). **A:** NFI-A; **B:** NFI-B; **C:** NFI-C; and **X:** NFI-X. Tissues from which mRNAs were prepared are indicated above each lane. Signals were quantified using a phosphorimager and normalized to the amount of S26 ribosomal protein mRNA present in each lane (data not shown).

spleen had consistently lower expression of all four NFI genes than the other tissues examined, and the four genes differed in their expression in the indicated tissues. At this gross level of analysis, the *Nfic* and *Nfix* genes appear most similar in their patterns of expression, whereas the patterns of the *Nfia* and *Nfib* genes also show some apparent similarities. The significance of these similarities in the expression patterns of these pairs of NFI genes is not yet clear (see Discussion). Because the four NFI genes exhibited overlapping expression patterns in adult tissues, we assessed their expression pattern in the developing mouse embryo by in situ hybridization.

Developmental Expression Patterns of the Four NFI Genes

Sections of mouse embryos from developmental stages 8.5 days postcoitum (dpc) to 16.5 dpc were hybridized in situ with ^{35}S-labeled antisense cRNA probes. The probes used were from the 3′ regions of the cDNAs isolated and should detect the known differentially spliced forms of NFI mRNAs with similar sensitivity (see Discussion). NFI-A was the only NFI family member detected before 10.5 dpc after 2 weeks of exposure of sections to nuclear track emulsion. By 9 dpc, NFI-A mRNAs were present in the heart, the septum transversum [which separates the pericardial and peritoneal cavities (Rugh, 1990)], and the anterior and posterior portions of the developing brain (data not shown). At 10.5 dpc, NFI-A transcripts were also detected in mesenchyme surrounding the posterior cardinal and vitelline veins, in the ventral portion of the neural tube, and at the distal margin of limb buds (data not shown). Also at this time, NFI-B mRNAs were detected in developing lung buds, and NFI-C gene transcripts were detected in cells surrounding the aortic arches and at a low level in dorsal root ganglia (data not shown). NFI-X mRNAs were not detectable within the level of sensitivity of the in situ technique at 10.5 dpc (data not shown).

At 11.5 dpc, all four NFI genes were expressed in partially overlapping patterns. In the central nervous system (CNS), NFI-A, NFI-B, and NFI-X mRNAs were detected in the neocortex region of the telencephalon, portions of the ventricular zone of the brain (Fig. 3A,B,X, marked t and m in A), and in the ependymal layer of the neural tube (data not shown). In contrast, NFI-C was expressed at very low levels in these regions of the CNS (Fig. 3C). In the peripheral nervous system, NFI-A was expressed at a higher level than NFI-C in dorsal root ganglia (Fig. 3A, three arrowheads and data not shown). In the mandibular arch (Fig. 3A, marked j), NFI-B and NFI-X were expressed throughout, but NFI-A and NFI-C were detected in more proximal mesenchyme (Fig. 3A,C). Each gene was detected in lung bud mesenchyme, but NFI-B was expressed at the highest level (Fig. 3). In limb buds, NFI-A was expressed in distal mesenchyme, but NFI-X was localized in central and proximal mesenchyme (data not shown). In gut mesenchyme, the levels of expression of NFI mRNAs were NFI-A > NFI-B > NFI-C (Fig. 3 and data not shown). In the liver, the relative levels were NFI-X > NFI-C > NFI-B > NFI-A (Fig. 3A, marked li). NFI-C mRNAs were detected around blood vessels such as the aortic arches and umbilical vessels (Fig. 3C, arrow). NFI-A gene transcripts were expressed highly in genital ridges, and NFI-X mRNAs were detected in skeletal muscle (myotomes; data not shown).

At 12.5–14.5 dpc, the expression patterns were the same as those described for 11.5 dpc with the following important additions. NFI-A, NFI-C, and NFI-X were expressed in developing skeletal muscles of the limbs and trunk (Fig. 4A,C,X and data not shown). NFI-B expression was detected predominantly around developing cartilage and in muscle connective tissue (Fig. 4B). NFI-A was expressed in mesenchyme around nasal sinuses (Fig. 4A, marked si), but NFI-B was detected in the sinus epithelium at a low level (Fig. 4B). Both NFI-A and NFI-B mRNAs were detected in the interdigital regions of limbs (Fig. 4A,B, marked hp). NFI-X expression was increased around umbilical blood vessels and was detected in gut mesentery and the genital tubercle (data not shown). NFI-A mRNAs were also detected in the gonad (Fig. 4A, marked g), which is consistent with its earlier expression in the genital ridge. NFI-B gene expression levels were the highest of

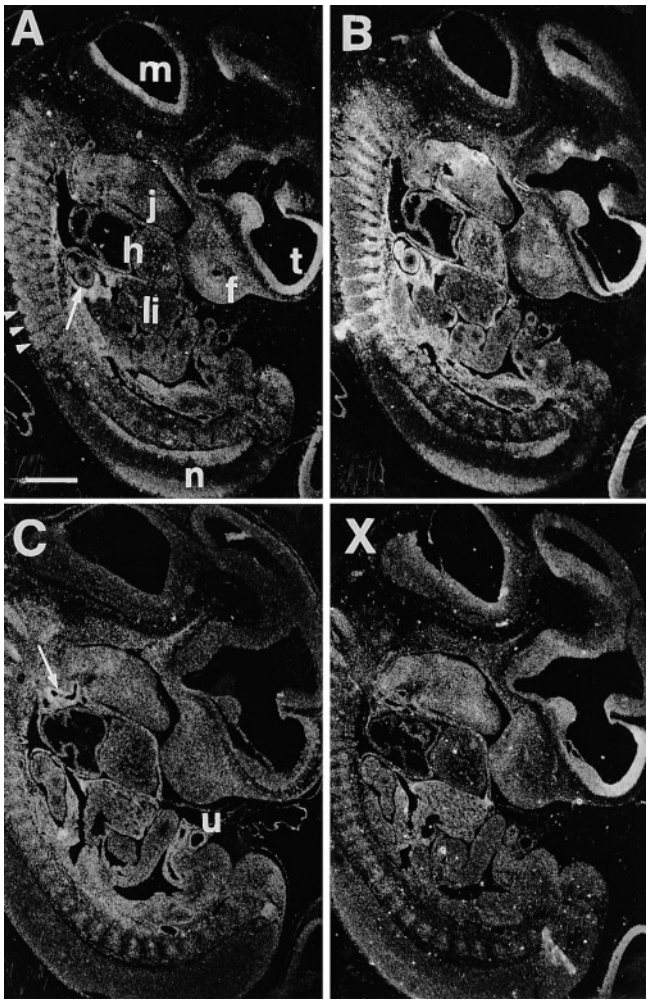


Fig. 3. NFI transcript localization at 11.5 dpc. Darkfield micrographs of parasagittal sections of a mouse embryo hybridized with (**A**) NFI-A, (**B**) NFI-B, (**C**) NFI-C, and (**X**) NFI-X antisense probes. The arrow in A points to the developing lung, a larger portion of which is seen in C and X. The arrowheads in A indicate several dorsal root ganglia. The arrow in C points to an aortic arch. f, frontonasal mass; h, heart; j, jaw; li, liver; m, metencephalon; n, neural tube; t, telencephalon; u, umbilical vessels. Scale bar = 500 μm.

the four genes in the lung (Fig. 4B, marked lu in A). In the CNS, NFI expression—with the exception of NFI-C—was very strong in the neocortex (Fig. 4A,B,X, marked c in A). NFI-B and NFI-X mRNAs were highly expressed in migrating neurons of the spinal cord and cerebellum (Fig. 4B,X and data not shown). In the peripheral nervous system, NFI-A and NFI-C were expressed in cranial ganglia (Fig. 4A,C, arrowhead in A) and dorsal root ganglia (data not shown). In the same ganglia, NFI-B was expressed in a punctate fashion that suggests it is localized in glial cells (Fig. 4B, marked arrowhead in A).

At 15.5–16.5 dpc, NFI-C and NFI-X were expressed in tooth primordia (Fig. 5C,X, small arrows in C). NFI-A was expressed at a high level in adipocytes of brown fat pads (Fig. 5A, filled arrow and data not shown) and in salivary glands (data not shown). Each of the four genes was expressed at some level around whisker follicles (Fig. 5A–X, marked w in A). In the CNS, NFI-B and NFI-X were highly expressed in pontine nuclei (Fig. 5B,X, marked open arrow in A), NFI-A was expressed at a lower level, and NFI-C was undetectable (Fig. 5A,C, open arrow in A). As was seen at earlier stages, NFI-A, NFI-B, and NFI-X were expressed at high levels in the cerebral cortex (Fig. 5A,B,X, marked c in A), whereas NFI-C was expressed only at relatively low levels (Fig. 5C).

In postnatal brain, each of the four NFI genes was expressed in granule cells in the cerebellum at birth and in the adult (data not shown). NFI-A and NFI-B were predominantly expressed in white matter of the cortex in the 2-week postnatal brain, suggesting they were found mainly in glial cells (Fig. 6A,B, marked w in A). NFI-X was expressed in gray matter, suggesting it is expressed predominantly in neurons in the cortex (Fig. 6X, marked g in A). In the hippocampus, NFI-A, NFI-B, and NFI-X are expressed at a higher level in the dentate gyrus than in the horn of Ammon cells (Fig. 6A,B,X, arrow in A). NFI-C was at the limit of detection of the in situ technique in this region (Fig. 6C). In the adult brain, NFI-B expression levels increased in the cortex, suggesting it is co-expressed with NFI-X in neurons (data not shown).

Transcriptional Activation Properties of the Four NFI Gene Products

Previous studies indicated that specific NFI proteins could activate transcription from cellular and viral promoters. However, some of these studies were performed before the discovery that NFI proteins are encoded by a multigene family, and the transcriptional modulation properties of the four mammalian NFI gene products have not been compared. To determine whether the expression pattern of NFI transcripts noted above might also result in differential transcriptional activation by NFI proteins, we assessed the ability of the four murine NFI proteins to modulate transcription from the glucocorticoid-dependent mouse mammary tumor virus (MMTV) promoter. The MMTV promoter was previously shown to contain an NFI binding site required for its expression (Miksicek et al., 1987; Bruggemeier et al., 1990). Activity of the MMTV promoter was measured in JEG-3 choriocarcinoma cells, which contain low levels of endogenous NFI proteins and were previously used to measure activation of the MMTV promoter by porcine NFI-C (Bruggemeier et al., 1990). Because JEG-3 cells contain only low levels of the glucocorticoid receptor, it was necessary to co-transfect a plasmid expressing the human glucocorticoid receptor to measure the activity of the MMTV promoter. Each of the four NFI gene products activated expression from the MMTV promoter, with NFI-B mediating an ~7-fold activation (Fig. 7A, lane 6) followed by, in decreasing order of activation, NFI-X (~5-fold, lane 10), NFI-C (~4-fold, lane 8), and NFI-A (~2-fold, lane 4).

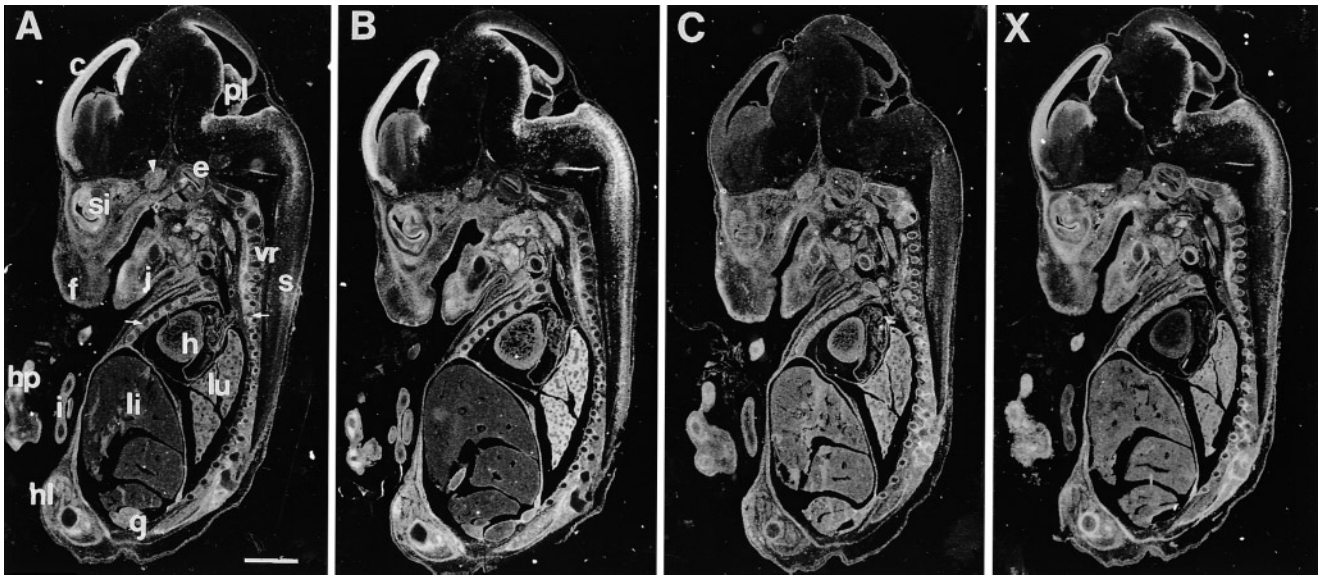


Fig. 4. NFI transcript localization at 14.5 dpc. Darkfield micrographs of parasagittal sections of a mouse embryo hybridized with (**A**) NFI-A, (**B**) NFI-B, (**C**) NFI-C, and (**X**) NFI-X antisense probes. The arrows in A points to ribs. The arrowhead points to a trigeminal ganglion. c, cortex; e, inner ear; f, frontonasal mass; g, gonad; h, heart; hl, hindlimb; hp, hindpaw; i, intestine; j, jaw; li, liver; lu, lung; pl, choroid plexus; s, spinal cord; si, nasal sinus; vr, vertebrae. Scale bar = 100 μm.

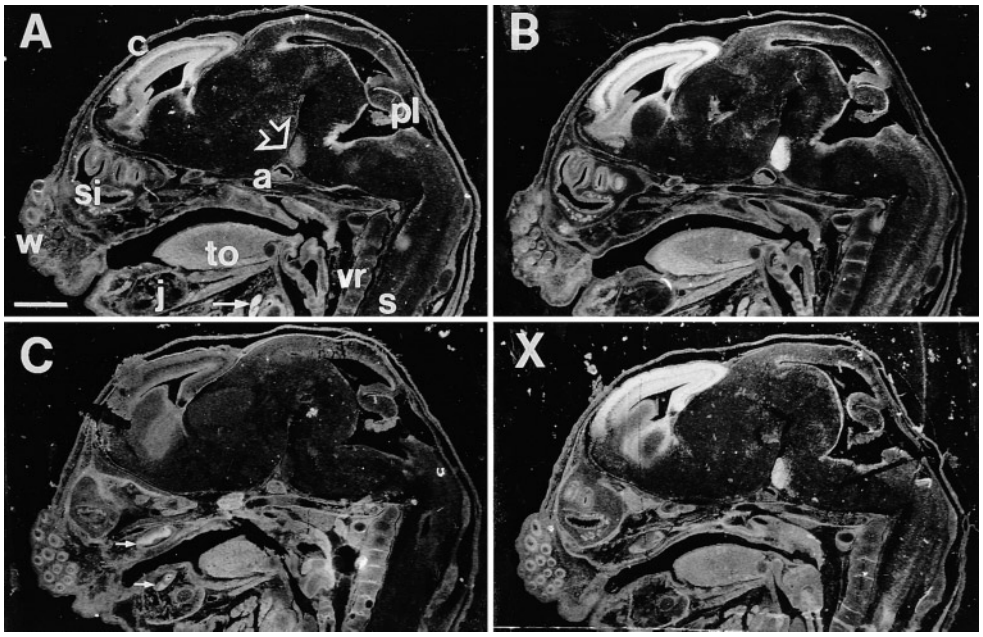


Fig. 5. NFI transcript localization at 16.5 dpc. Darkfield micrographs of parasagittal sections of a mouse fetal head region hybridized with (**A**) NFI-A, (**B**) NFI-B, (**C**) NFI-C, and (**X**) NFI-X antisense probes. The small arrow in A indicates adipocytes below the jaw. The open arrow in A points to a nucleus in the pons region of the brain. The arrows in C indicate tooth primordia. a, anterior pituitary; c, cerebral cortex; j, jaw; pl, choroid plexus; s, spinal cord; si, nasal sinus; to, tongue; vr, vertebrae; w, whisker follicles. Scale bar = 500 μm.

These differences are not due to differences in the level of expression of the proteins, because Western blot analysis (Fig. 7B) and DNA binding assays (not shown) showed similar expression levels of the four NFI proteins. In addition, the transfections were performed using saturating amounts of the NFI expression plasmids (not shown). As expected, all activity from the MMTV promoter was glucocorticoid dependent (Fig. 7A, − versus + Dex). These differences in the ability of these specific NFI gene products to activate expression from the MMTV promoter suggest that similar differences might exist in the response of cellular promoters

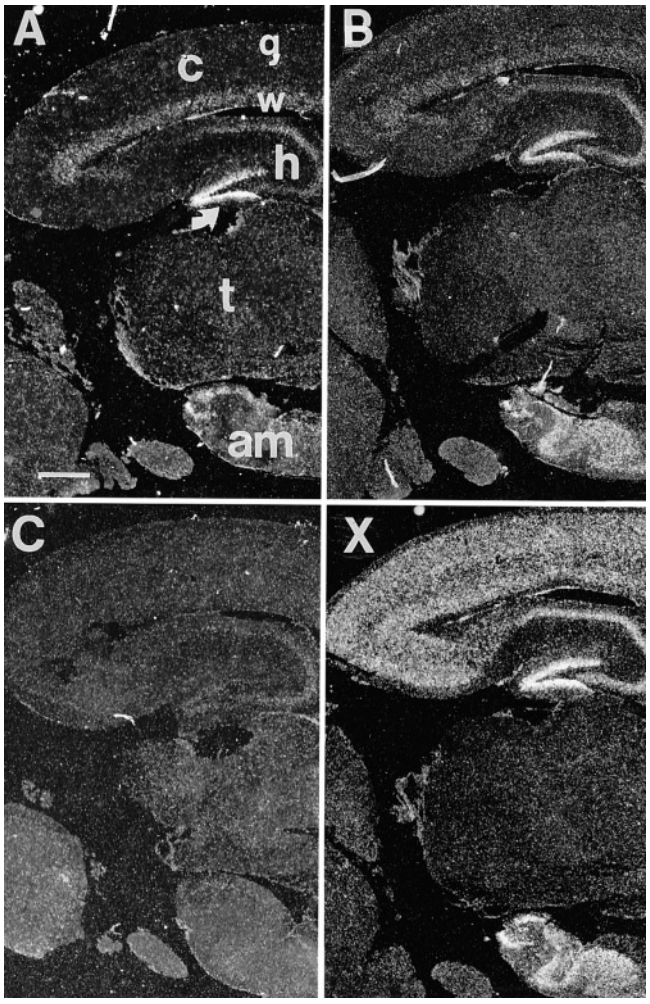


Fig. 6. NFI transcript localization in gray and white matter in postnatal brain. Darkfield micrographs of parasagittal sections of a 2-week postnatal brain hybridized with (**A**) NFI-A, (**B**) NFI-B, (**C**) NFI-C, and (**X**) NFI-X antisense probes. The arrow in A points to the dentate gyrus. am, amygdala; c, cerebral cortex; g, gray matter; h, hippocampus; t, thalamus; w, white matter. Scale bar = 500 μm.

to the NFI proteins. Given the expression patterns of the NFI genes seen during murine development, it seems likely that the four genes play distinct roles in tissue-specific gene expression during cell growth and differentiation.

DISCUSSION

These studies provide the first detailed analysis of the expression patterns and transcriptional activation properties of the NFI family of transcription/replication proteins. The four cDNAs obtained provide a useful resource to begin to understand how the diverse NFI gene products differ in their transcriptional modulation properties. The observation that the four NFI genes are expressed in distinct patterns in postnatal (Figs. 2, 6) and embryonic (Figs. 3–5) tissues, together with the differences seen in the transcriptional activation of the MMTV promoter (Fig. 7A), suggest that changes in the relative levels of the different NFI proteins could have a profound effect on the tissue-specific expression of cellular NFI-dependent genes.

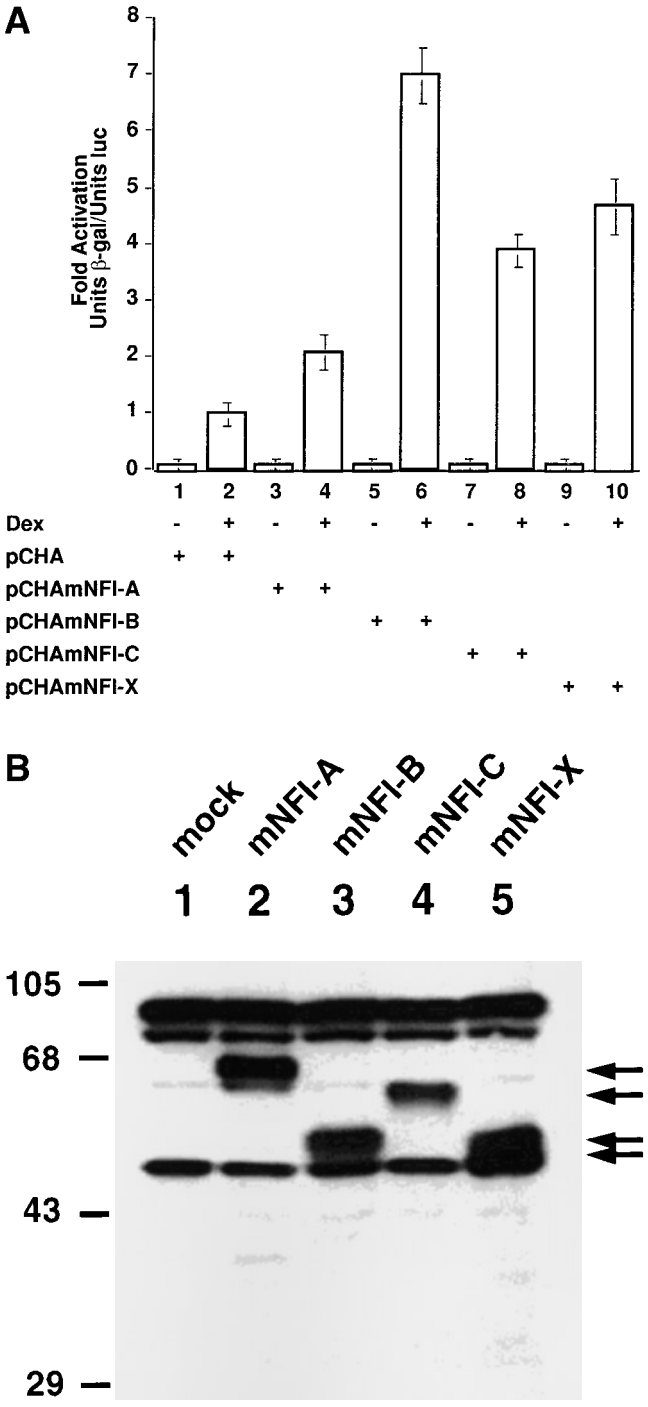
Features of the Murine NFI Genes

The ability to amplify a 486 bp region within the DNA-binding domains of all four murine NFI genes from genomic DNA demonstrates that the genomic structure of this region is similar to that seen previously for the porcine NFI-C gene (Meisterernst et al., 1989) and the four human NFI genes (Kulkarni and Gronostajski, 1996). In each of the NFI genes, the N-terminal DNA-binding domain is encoded primarily by a single exon (exon 2 in the genes analyzed). This conservation in genomic structure between the four genes suggests that they probably arose by multiple duplication events during vertebrate evolution. This model has been strengthened by the identification of a single NFI gene in *Caenorhabditis elegans* (NCBI gi 687804), which contains a 3′ splice site at the beginning of the DNA-binding domain that is identical in position to splice sites at the second exons of the porcine NFI-C and mouse NFI-A genes (Gronostajski, R.M. Unpublished data, 1996). Surprisingly, there are four additional introns within the DNA-binding domain region of the *C. elegans* NFI gene that are absent from all vertebrate NFI genes. If *C. elegans* contains only a single NFI gene (as is indicated by PCR and Southern analysis; Gronostajski, R.M. Unpublished data, 1996), it would suggest either that these four introns were excised from the gene before the duplications that created the vertebrate genes or that introns have been inserted into the *C. elegans* gene at some point in evolution. Whichever explanation is correct, it is clear that the NFI gene has been conserved through at least 500 million years of evolution and that the four NFI genes have been highly conserved as a family from chickens to humans.

Differential Splicing of Transcripts of the NFI Genes

Previous studies had demonstrated that transcripts for each of the four vertebrate NFI genes are differentially spliced, with more than 18 isoforms of NFI gene products now identified (Santoro et al., 1988; Meisterernst et al., 1989; Inoue et al., 1990; Rupp et al., 1990; Kruse et al., 1991; Apt et al., 1994; Kruse and Sippel, 1994a; Nebl and Cato, 1995). Many of these differentially spliced NFI mRNAs have been detected only in individual species, but the recent observation that several splice variants are present in multiple species indicates that most or all of the isoforms may be present in the mouse (Kruse and Sippel, 1994a). For this reason, it was important to use in situ probes that would detect most or all of the known isoforms of NFI mRNAs with similar sensitivity. The NFI-A and NFI-B probes used were completely contained within the 3′UTRs of their respective mRNAs and should detect

all splice variants where the 3′UTR has been identified (Inoue et al., 1990; Kruse and Sippel, 1994a). The NFI-X probe, while extending into the coding region, should also detect the known NFI-X isoforms with equal sensitivity (Apt et al., 1994; Nebl and Cato, 1995). The NFI-C probe extends through a region of differential splicing, but contains at least 456 bp of homology to each of the known isoforms, and has complete homology (767 bp) to the three major NFI-C/CTF isoforms identified (Santoro et al., 1988; Meisterernst et al., 1989; Altmann et al., 1994). Thus, these probes appear suitable for the global analysis of NFI gene expression shown here. However, because the relative levels of the known splice variants of NFI mRNAs have been characterized in only a few cell lines (Apt et al., 1994; Kulkarni and Gronostajski, 1996), it will be of great interest in the future to use isoform-specific probes to determine whether the differential splicing of NFI mRNAs is regulated during development and whether particular splice variants may play essential roles in specific tissues.



Spatial and Temporal Expression of NFI Transcripts in Mouse Embryos

Although the expression patterns of the mouse NFI genes are complex, some prototypical patterns have been noted by comparison of the four genes. The two major types of developmental patterns seen for the four genes are preferential expression of a single family member in a tissue at a specific time point, and coordinate expression of two or more family members in a tissue or cell type—especially concordant expression of NFI-C and NFI-X in connective tissue (Fig. 4C,X). The NFI-C and NFI-X genes appear to be the two family members most closely related as assessed by sequence comparison of the four genes (data not shown) and their co-localization to chromosome region 19p13.3 in humans (Qian et al., 1995). Although the pathway of gene duplications that generated the NFI gene family is unknown, these co-expression data (together with co-localization in the genome) may indicate that NFI-C and NFI-X were generated by a relatively recent duplication during vertebrate evolution. To address this potential pathway of genome development, it will be necessary to assess the number, sequence, and expression pattern of NFI genes in more distant organisms. It will then be of particular interest to determine whether the expression patterns of the NFI genes in less complex organisms correlate with that seen in the mouse or whether major changes have occurred in the expression

Fig. 7. Differential activation of the MMTV promoter by the four murine NFI proteins. **A:** Control vector pCHA (0.5 μg, lanes 1–2) and vectors expressing each of the four murine HA-epitope tagged NFI proteins (0.5 μg each; pCHAmNFI-A, lanes 3–4; pCHAmNFI-B, lanes 5–6; pCHAmNFI-C, lanes 7–8; pCHAmNFI-X, lanes 9–10) were co-transfected into JEG-3 cells with the MMTV-βgal reporter (5 μg), internal control SV40-luciferase vector (2.5 μg), and hGR-expression vector (2.5 μg). Cells were cultured for 48 hr in the absence or presence of dexamethasone (− or + Dex). β-galactosidase activity was normalized to luciferase levels and expressed as fold activation over the control vector (lane 2). The bars represent the mean and range of six measurements from three independent transfections using different DNA preparations. **B:** Expression levels of the four NFI proteins. JEG-3 cells were transfected with 2.5 μg of NFI cDNA expression vectors. Lane 1, mock transfected; lane 2, pCHAmNFI-A; lane 3, pCHAmNFI-B; lane 4, pCHAmNFI-C; lane 5, pCHAmNFI-X. Whole cell extracts were analyzed on a 7.5% SDS-PAGE gel, transferred to Immobilon-P membrane, and probed with anti-HA antibody. Numbers at left indicate size markers (*M*r x10^{-3}), and arrows on the right indicate HA-tagged NFI polypeptides of the predicted size for each cDNA.

pattern of the gene family during speciation. Any changes observed in NFI expression would be useful in assessing the role of NFI proteins in vertebrate body plan development.

NFI Expression in Muscle

Previous studies suggested that NFI proteins may play an important role in skeletal muscle development. Binding sites for NFI proteins were co-selected with binding sites for the myogenic regulator, myogenin, during immunoprecipitation of myogenin-DNA complexes from libraries of random binding sites (Funk and Wright, 1992). The co-selection of NFI binding sites in these studies, together with evidence for enhanced binding of myogenin to compound myogenin-NFI binding sites (Funk and Wright, 1992) and the presence of apparent NFI binding sites in the promoters of the myogenin (Edmondson et al., 1992) and muscle-specific phosphofructo-kinase genes (Darville et al., 1992), suggest a role for NFI proteins in myogenic regulation. Whereas NFI-A expression appears high in hindlimb muscle at 12.5 dpc (Fig. 4A, marked hl), the overlapping expression of the NFI genes in developing skeletal muscle after day 12.5 (Fig. 4A, marked hl, Fig. 5, and data not shown) makes it difficult to assess which NFI gene product would be most important in muscle development. For example, none of the well-characterized myogenic regulators (e.g., MRF4, Myf-5, Mef2, and MyoD) are expressed in precisely the same spatial and temporal fashion as any of the four NFI genes (Bober et al., 1991; Ott et al., 1991; Edmondson et al., 1994; Asakura et al., 1995). Thus, it may be necessary to disrupt one or more of the NFI genes in the mouse to determine the precise role of the NFI gene family in muscle development.

NFI Expression in the Nervous System

The remarkable expression of NFI-A, NFI-B, and NFI-X in developing neocortex suggests an important role for these genes in brain development (Figs. 3A,B, 4A,B,X, 5A,B,X). Although previous studies have indicated that NFI-A expression was high in adult cerebellum (Inoue et al., 1990), our data indicate that NFI-A is expressed both early and late in cortical development (Figs. 3A–5A). In addition, NFI-B and NFI-X are also expressed at high levels in the developing neocortex (Figs. 3B, 4B,X, 5B,X) and midbrain (Figs. 4, 5, regions around pl in A). Although this expression pattern of the NFI genes suggests an important role in brain development, there are few known target genes for NFI that are expressed in the brain at these times. The best characterized NFI-responsive genes in the brain are the myelin basic protein (MBP) gene and the mid-sized neurofilament gene [NF(M)], both of which are expressed primarily postnatally in glial cells and neurons, respectively (Verity and Campagnoni, 1988; Lee et al., 1992). Although it is difficult to correlate the embryonic expression of the NFI genes with the expression of these brain-specific genes, the expression of NFI-A, NFI-B, and NFI-X transcripts in the white matter of the 2-week cortex is consistent with a role in the postnatal activation of the MBP promoter (Fig. 6A,B,X, marked w in A). Thus, these findings support previous studies that showed both a requirement for an NFI-binding site for the maximal activity of the MBP promoter in vitro (Tamura et al., 1988) and activation of the MBP promoter by NFI-A in a transfected glial cell line (Inoue et al., 1990). It will be of particular interest to determine if the four NFI gene products described here (Fig. 1) differ in their abilities to activate the MBP promoter or other brain-specific promoters.

Whereas the physiologic role of NFI proteins in the brain is most likely to regulate the expression of brain-specific genes, a number of neurotropic viruses also contain NFI-binding sites essential for their replication and transcription. Several studies have indicated that an NFI-binding site in the JC virus early region is important for both the brain-specific transcription (Amemiya et al., 1992; Kumar et al., 1993; Krebs et al., 1995) and replication (Sock et al., 1991) of JC virus. Also, a cluster of multiple NFI binding sites in the immediate early region of the human and simian cytomegaloviruses (CMV) have been proposed to play a role in viral replication (Hennighausen and Fleckenstein, 1986; Jeang et al., 1987). Although there is no direct evidence that NFI proteins play an essential role in CMV infection, some of the regions of NFI expression in the CNS correspond to tissues affected by congenital human CMV infection, including the cerebral cortex (Figs. 4A–X, 5A–X, marked c in Figs. 4A and 5A) and regions of the midbrain surrounding the choroid plexus (Fig. 4A-X, 5A-X, marked pl in Figs. 4A and 5A) (see (Koedood et al., 1995) for review). The NFI cDNAs isolated here provide important tools for future studies to test for a role of specific NFI gene products in viral neuropathogenesis.

NFI Expression in Liver

In developing liver, by 11.5 dpc NFI-X and NFI-C transcripts are detected at moderate levels, whereas NFI-A and NFI-B transcripts are low (Fig. 3A–X, marked li in A). Such distinct expression of the four NFI genes in liver continues through 14.5 dpc (Fig. 4A–X, marked li in A). However, all four genes appear highly expressed in the adult liver (Fig. 2). This change in the expression pattern of the NFI genes may be related to the known coordinate activation and repression of the liver-specific serum albumin and α-fetoprotein (AFP) genes. Albumin is first expressed at 10.5 dpc and continues to be expressed throughout adult life. Conversely, the AFP promoter is most active during fetal development and is inactive postnatally (Tilghman and Belayew, 1982). Mutational analysis of the albumin promoter indicates that NFI proteins cooperate with hepatic nuclear factor 3 (HNF3) to stimulate albumin expression (Bois-Joyeux and Danan, 1994). In contrast, NFI proteins appear to repress AFP expression by a direct competition between NFI proteins and HNF1 for

overlapping binding sites in the AFP promoter (Bernier et al., 1993). Our data are consistent with a model in which NFI-X and NFI-C levels at 11.5 dpc are sufficient to maintain the serum albumin promoter in an active state, but are not sufficient for repression of the AFP promoter. However, in adult tissues in which all four genes are expressed at high levels (Fig. 2), there would be sufficient levels of NFI to repress AFP expression. These data are also consistent with a model in which the four NFI gene products differ in their activities on the two promoters (e.g., high levels of NFI-A proteins postnatally could repress AFP expression but have no effect on albumin expression). It is now possible to distinguish between these models by overexpression of the different NFI cDNAs in hepatocytes or transgenic mice and measuring the effect on albumin and AFP expression (Bois-Joyeux and Danan, 1994).

NFI-A Expression in Fat Pads

In addition to its elevated expression in the cortex and other sites, high levels of NFI-A transcripts are present in adipocytes of brown fat pads at 15.5–16.5 dpc (Fig. 5, small horizontal arrow at bottom of A and data not shown). It was shown previously that an NFI binding site is required for the adipocyte-specific expression of the P2 gene (Graves et al., 1991). However, the developmental stage in which NFI is required and the specific NFI gene product that mediates the expression of the P2 gene remains undetermined. The high levels of NFI-A transcripts in fat pads at 15.5 dpc support a model in which an NFI-A gene product may be involved in the expression of the P2 gene and other genes at this stage in adipogenesis.

Transcriptional Activation Properties of the Four NFI Gene Products

The four murine NFI gene products isolated activate expression of the MMTV promoter by differing degrees (Fig. 7A). Previous studies indicated that NFI proteins possess a two-domain structure, with an N-terminal DNA binding domain that is highly homologous (but distinct) between the four genes and C-terminal domains that are much less homologous (Rupp and Sippel, 1987; Meisterernst et al., 1989; Mermod et al., 1989; Gounari et al., 1990). The different activation potentials of the four NFI proteins seen here appear to be mediated by differences in the C-terminal domain, as deletion constructs of NFI-B and NFI-C containing only the N-terminal DNA binding domain activate the MMTV promoter to a similar low level ($<$2-fold, data not shown). These findings support previous studies that suggest that transcriptional modulation properties of the human NFI-C and NFI-X proteins reside in their C-terminal domains (Mermod et al., 1989; Apt et al., 1993).

The four NFI gene products identified here represent only a small subset of the known differentially spliced isoforms of NFI (Apt et al., 1994; Kruse and Sippel, 1994a); the multiple splice variants derived from the same NFI gene have been shown to have widely different transcriptional modulation properties (Santoro et al., 1988; Altmann et al., 1994; Apt et al., 1994; Nebl and Cato, 1995). Thus, it is likely that the differences detected here on the MMTV promoter do not reflect the maximal differences between the transcription modulation properties of NFI family members and may not reflect physiologically relevant differences in the function of NFI family members. Future studies will be needed to determine which specific NFI family member or members is (are) most important in vivo for the growth and development of specific tissues. In addition, it should be noted that the expression patterns detected here (Figs. 2–6) reflect the sum total of all differentially spliced mRNAs from each of the four NFI genes analyzed and may not represent the levels of the specific cDNAs used in the transactivation studies (Fig. 7).

Currently, the molecular basis for the differential activation properties of the four NFI gene products studied here is unclear. Previous studies showed that one NFI-C gene product (NFI-C/CTF1) contains a region homologous to the C-terminal domain of RNA polymerase II (CTD) and that this region of NFI-C physically interacts with components of the basal transcriptional machinery (Altmann et al., 1994; Kim and Roeder, 1994; Xiao et al., 1994). This interaction has been proposed to increase the rate of transcription by enhancing the recruitment of transcription proteins to promoters containing NFI binding sites. However, neither the NFI-B nor the NFI-X proteins isolated in this study contain a CTD-like repeat, yet they can activate transcription. Also, a known splice variant of the NFI-C/CTF proteins, CTF-5, is a potent transcriptional activator but lacks the CTD-like repeat (Wenzelides et al., 1996). These data indicate that either additional unknown protein domain(s) on the NFI proteins can substitute for the CTD-repeat in promoting interactions with basal transcription factors or that the NFI gene products may differ in the molecular basis of their transcriptional activation. Finally, although NFI-A stimulated the MMTV promoter only weakly in JEG-3 cells (Fig. 7A), an identical NFI-A protein activated expression of the MBP promoter 5- to 8-fold in NG108-15 glial cells (Inoue et al., 1990). Thus, it appears likely that the activation potentials of the different NFI gene products are promoter or cell-type dependent. These apparent differences in the activation potential of the NFI gene products, together with the diverse pattern of NFI expression we report here, suggest that cell-type specific modulation of transcription by the NFI proteins may play an important role in mammalian development.

EXPERIMENTAL PROCEDURES

Cloning of cDNAs for the Four Murine NFI Genes and Isolation of Gene-specific Probes

Degenerate oligonucleotides Deg1 and Deg2 (Kulkarni and Gronostajski, 1996) were used to amplify 427 bp regions of the DNA binding domains of the four NFI genes from mouse genomic DNA. The primers were

derived from the most conserved regions of the known vertebrate NFI genes, were designed to be contained within a single exon of the previously cloned porcine NFI-C gene (Meisterernst et al., 1989), and were identical to those used previously to amplify cDNAs of the four chicken and human NFI genes (Rupp et al., 1990; Kulkarni and Gronostajski, 1996). PCR fragments were cloned into the pBSIIKS+ vector (Stratagene, LaJolla, CA) and sequenced. Gene-specific oligonucleotide probes (Fig. 1) were made from the most divergent regions of the four NFI PCR fragments. Oligonucleotides were labeled using polynucleotide kinase ($\sim 10^9$ cpm/mg) and their specificity was checked by hybridization to dot-blots containing known amounts of the four cloned NFI PCR products. The probes were used to analyze multi-tissue Northern blots (Clontech, Palo Alto, CA) and to screen mouse liver and skeletal muscle cDNA libraries (Clontech) using standard techniques. Multiple cDNA clones for each NFI gene were restriction mapped, and the largest clones were subcloned and sequenced. Gene-specific probes for in situ hybridization were generated from 3′ regions of the cloned cDNAs (Fig. 1).

Transient Transfection Assays and Plasmid Constructs

JEG-3 choriocarcinoma cells were cultured in α-MEM medium containing 10% fetal bovine serum. Cells were transfected using calcium phosphate and the indicated amounts of DNA as described (Gorman, 1985). Transfections were performed in quadruplicate using duplicate precipitates for each point, and all results were confirmed by multiple independent experiments. MMTV promoter expression was measured from a derivative of the pMAMNeo vector (Clontech) containing the MMTV promoter driving expression of the β-galactosidase (β-gal) gene (Hall et al., 1983). β-gal activity is expressed relative to the activity of an SV40-luciferase vector present as an internal control (pGL2-Control, Promega, Madison, WI). Coding regions of the NFI cDNAs were cloned and expressed in the pCMVβ vector containing the CMV immediate early promoter (MacGregor and Caskey, 1989). Because the NFI-A and NFI-X cDNAs terminated 3 bp and 9 bp 3′ to their respective initiation methionines (as compared to chicken, human, porcine, and hamster NFI cDNAs), the peptide sequences M and MYS were added to the N-termini of these two proteins, respectively, by PCR mutagenesis. Each NFI coding region was subcloned as a fusion protein with an N-terminal tag from the influenza virus hemagglutinin protein (HA-tag, **YPYDVPDYA**) (Field et al., 1988). No differences between the DNA-binding or transactivation functions of wild-type and HA-tagged NFI proteins were observed.

Western Blot Analysis

JEG-3 cells transfected with the NFI cDNA expression vectors were pelleted by centrifugation; samples were dissolved in 1× Laemmli buffer (Laemmli, 1970), boiled for 15 min, analyzed on a 7.5% SDS-polyacrylamide gel, and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA). The blots were blocked and hybridized with α-HA antibody (Boehringer Mannheim, Indianapolis, IN) as per manufacturer's instructions, and HA-tagged proteins were detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL).

In Situ Hybridization of Tissue Sections

The protocol used to fix and embed C57BL/6xDBA/2 embryos and postnatal tissues was described previously (Lyons et al., 1990). To reliably distinguish each of the four NFI gene products from other members of the multigene family, we used antisense probes from the 3′ coding and noncoding regions of the NFI mRNAs that differ between the four NFI genes (Fig. 1). The specificity of each probe was verified by hybridization to Northern blots, and probe lengths and emulsion exposure times were controlled to allow comparison of the relative expression levels of the four genes. The probes were designed to detect the known spliced NFI transcripts with similar efficiency. Sense control probes were also used as negative controls in all experiments. All sense control probes resulted in a low, background level of silver grains (data not shown).

The cRNA transcripts were synthesized according to manufacturer's instructions (Stratagene) and labeled with ^{35}S-UTP (>1000 Ci/mmol; Amersham). cRNA transcripts larger than 100 nt were subjected to alkali hydrolysis to give a mean size of 100 nt for efficient hybridization. Sections were hybridized, treated with RNase A, exposed to nuclear track emulsion, and developed as described previously (Lyons et al., 1990). Slides were counterstained lightly with toluidine blue and analyzed using both lightfield and darkfield optics. Embryonic structures were identified with the help of atlases (Rugh, 1990; Kaufman, 1992; Schambra et al., 1992).

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