Promoter-specific differences in the function of transcription factors play a central role in the regulation of gene expression. We have measured the maximal transcriptional activation potentials of nuclear factor I (NFI) proteins encoded by each of the four identified NFI genes (NFI-A, -B, -C, and -X) by transient transfection in JEG-3 cells using two model NFI-dependent promoters: 1) a simple chimeric promoter containing a single NFI-binding site upstream of the adenovirus major late promoter (NFI-Ad), and 2) the more complex mouse mammary tumor virus long terminal repeat promoter. The relative activation potentials for the NFI isoforms differed between the two promoters, with NFI-X being the strongest activator of NFI-Ad and NFI-B being the strongest activator of the MMTV promoter. To determine if these promoter-specific differences in activation potential were due to the presence of glucocorticoid response elements (GREs), we added GREs upstream of the NFI-binding site in NFI-Ad. NFI-X remains the strongest activator of the GRE-containing simple promoter, indicating that differences in relative activation potential are not due solely to the presence of GREs. Since NFI proteins bind to DNA as dimers, we assessed the activation potentials of NFI heterodimers. Here, we show that NFI heterodimers have intermediate activation potentials compared with homodimers, demonstrating one potential mechanism by which different NFI proteins can regulate gene expression.

The expression of RNA polymerase II-dependent genes is mediated by a complex set of DNA-protein and protein-protein interactions. General transcription factors directly interact with the RNA polymerase holoenzyme to form a transcription-competent complex which is capable of a basal level of transcription (see Refs. 1 and 2, for reviews). Efficient gene transcription occurs when promoter-specific regulatory proteins (transcriptional activators and repressors) bind to DNA sequences in promoters and enhancers and interact directly, or via adapter proteins, with components of the basal machinery (Refs. 3–6; and see Refs. 7 and 8, for reviews). Transcriptional activation occurs when promoter-specific regulatory proteins (transcriptional activators and repressors) bind to DNA sequences in promoters and enhancers and interact directly, or via adapter proteins, with components of the basal machinery (Refs. 3–6; and see Refs. 7 and 8, for reviews). Transcriptional activators are modular in nature containing separable and functionally distinct DNA binding and activation domains (Refs. 9 and 10; see Ref. 11, for review). Activation domains have been grouped into three classes depending on their amino acid composition: acidic, glutamine-rich, or proline-rich (9, 12). We are studying the nuclear factor I (NFI) family of site-specific DNA-binding proteins which, based on the amino acid composition of NFI-C proteins, is grouped into the proline-rich class of activators (13).

NFI was initially identified as a host-encoded protein required for the efficient initiation of adenovirus (Ad) replication in vitro (14) and was later shown to be required for the correct expression of numerous cellular and viral genes. Cloning of cDNAs encoding NFI proteins from various species (13, 15–17) 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 contain a highly conserved NH2-terminal 220 amino acid region, which mediates DNA binding, dimerization, and the initiation of Ad replication (18–20). NFI proteins bind to DNA as both homo- and heterodimers and recognize the consensus binding site, TTGGC(N5)GCCAA with the same apparent affinity (21, 22). However, considerable variation occurs within the COOH-terminal domains of the NFI proteins which likely encode distinct transcription modulation domains. Additional variation between NFI proteins is generated through differential splicing of transcripts from each of the four genes (23).

The existence of 4 different NFI genes in vertebrates, their differential expression during mouse development (24), and the regulated expression of NFI-dependent genes expressed in multiple organs including brain (25, 26), liver (27), muscle (28, 29), and other terminally differentiated tissues (30), argues in favor of diverse transcription modulation properties for NFI proteins. Different NFI-C isoforms isolated from HeLa cells and porcine liver have different transcriptional activation potentials (13, 15). The precise mechanism of NFI-mediated activation is unknown, however, direct interactions between one NFI-C protein (CTF1) and components of the basal transcriptional machinery have been reported (31–33). This interaction was shown to be dependent on a sequence motif related to the COOH-terminal heptapeptide repeat (CTD) of RNA polymerase II. However, this is unlikely to be the only mechanism by which NFI proteins activate transcription as some NFI proteins lacking a CTD repeat are potent activators in both yeast (34, 35) and mammalian cells (24, 36).

Although it is widely accepted that NFI proteins function as...
transcription modulators, it is not clear if they exhibit promoter-specific differences in activation potential. To address this question, and to better understand the transcription modulation properties of NFI proteins, we performed transient transfection experiments using two model NFI-dependent reporter constructs. Here, we demonstrate that NFI isoforms representing each of the 4 murine NFI genes exhibit a broad range of transactivation potentials. Our data also show that activation potentials are modulated in a promoter-specific manner, determined by the COOH-terminal regions of the NFI proteins.

**MATERIALS AND METHODS**

**Plasmid Constructs**—The reporter plasmids, pNFIβ-gal and pNFIβ-gal, are derivatives of the pB series of vectors which were described previously (37). The bacterial chloramphenicol acetyltransferase gene of pB and pBBF was removed by digestion with BstI and repair of the ends with Klenow polymerase. The bacterial β-galactosidase gene (β-gal) was isolated by digesting pCMVβ-gal (Stratagene) with NotI and repair of the overhanging ends with Klenow polymerase. The blunt NotI β-gal fragment was cloned into pB and pBBF creating pNFIβ-gal and pNFIβ-gal, respectively. Orientation was verified by restriction enzyme digestion. pNFIβ-gal differs from pNFIβ-gal by presence of a single NFI-binding site cloned immediately upstream of the Ad major late promoter (51 to +33) promoter element. The blunted pNFIβ-gal plasmid pNFIGRE-gal was cloned by generating the synthetic oligonucleotide (CTAGTGTTAGGAGTGGTCCTGGGCGCTTAGAGA- TTGTCTCTTAG) which consists of 4 GRE half-sites (in bold) into the XbaI site of pNFIβ-gal. pMVTβ-gal was created by cloning the HindIII/BamHI fragment of pMAMNeoβ-gal (CLONTECH) into HindIII/BamHI digested pBSIIKS+(Stratagene). NFI effector plasmids pCHNFI-B, pCHNFI-C, and pCHNFI-X have been described previously (24) and express murine proteins homologous to chicken NFI-B2, -C2, and human NFI-X2 (23). pCHNFI-A is identical to the NFI-A effector plasmid described previously (24) except that it has a 92-nucleotide deletion at residue 473 of the coding sequence, which changes the reading frame and generates a murine protein homologous to chicken NFI-A4 (23).

Plasmid pCHNFI-B-235, which expresses the DNA-binding domain of NFI-B, pCHNFI-B, pCHNFI-C, and pCHNFI-X have been described previously (24) and generates a murine protein homologous to chicken NFI-A4 (23). Each NFI protein contains an NH2-terminal human influenza virus hemagglutinin (HA) antibody (Boehringer Mannheim), binding reactions were carried out for 30 min on ice, followed by the addition of 1 μl of the anti-HA antibody (200 μg/ml) and further incubation for 20 min at room temperature. As a control, binding reactions not incubated with antibody, were treated with 1 μl of PBS and incubated for 20 min at room temperature. The DNA-protein complexes were resolved as described in Results and analyzed using a Molecular Dynamics model 4000 PhosphorImager.

**Immunocytochemistry**—To detect the intracellular location of the HA-tagged NFI proteins, HeLa cells were grown on coverslips, transfected with vectors expressing the NFI protein or control vectors, cultured for 48 h, and fixed in chilled methanol at −20 °C for 15 min. Fixed cells were blocked for 1 h at room temperature with 3% bovine serum albumin, 0.1% Tween 20 in PBS, incubated with the anti-HA antibody in PBS for 45 min, washed in PBS, incubated for 45 min with fluorescein isocyanate-conjugated secondary antibody, washed in PBS, the coverslips were mounted with Vectashield (Vector Laboratories), the cells were examined using a Nikon fluorescence microscope and the images were captured with an Oncor imaging system (Cleveland Clinic Fluorescent Microscopy Core). For control staining, the specific antibody solution was replaced by PBS alone.

**RESULTS**

**NFI Proteins Exhibit Promoter-specific Differences in Their Maximal Activation Potentials**—We have recently reported the cloning of NFI cDNAs from each of the 4 murine NFI genes, analyzed their embryonic and postnatal expression patterns, and demonstrated their ability to activate the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter in JEG-3 cells (24). To extend these studies we have used transient transfection assays to determine if the four NFI gene products exhibit promoter-specific differences in activation potential. The NFI effector plasmids and NFI-dependent reporter constructs used in co-transfection experiments are shown in Fig. 1A. NFI effector plasmids contain the human cytomegalovirus (CMV) immediate early promoter/enhancer (40) expressing murine proteins homologous to chicken NFI-A4, NFI-B2, NFI-C2, and human NFI-X2 (23). Each NFI protein contains an NH2-terminal human influenza virus hemagglutinin (HA) epitope tag (41) for ease of detection. The NFI-dependent promoters used are a simple chimeric promoter, pNFIβ-gal and the complex viral pMMTVβ-gal promoter reporter construct. The reporter plasmid pNFIβ-gal is derived from the chimeric pB series of vectors which have been described previously (37) and contains a single NFI-binding site upstream of the adenovirus major late promoter (−51 to +33) driving expression of the bacterial β-gal gene. pMMTVβ-gal contains the glucocorticoid-inducible MMTV promoter driving expression of β-gal. The MMTV promoter has previously been shown to contain an NFI-binding site required for its function (42, 43). Transfection experiments were carried out in JEG-3 human choriocarcinoma cells, which contain low levels of endogenous NFI pro-
teins and have previously been used to measure activation of the MMTV promoter by porcine NFI-C (43).

To assess the maximal transactivation potential of each of the NFI isoforms, increasing amounts of each effector plasmid were transfected into JEG-3 cells. With both NFI-dependent promoters, increasing amounts of each effector plasmid caused a simple monotonic increase in expression up to apparent saturation, showing no squelching at high levels of effector plasmid (Fig. 1B, i and ii). The maximal level of reporter expression differed for each of the isoforms and was promoter-specific. NFI-X shows the strongest activation of pNFIβ-gal (~17-fold, Fig. 1B, panel i), followed by, in decreasing order of activation, NFI-B (~14-fold), NFI-C (~9-fold), and NFI-A (~3-fold). In contrast, NFI-B is the most potent activator of pMMTVβ-gal (Fig. 1B, panel ii) with maximal activation of ~13-fold, followed by NFI-X (~11-fold), NFI-C (~6-fold), and NFI-A (~3-fold). The fold activation is shown only in the presence of dexamethasone as MMTV promoter activity in the absence of hormone induction is undetectable (see Fig. 2B, for example). Differences in activation potential of each NFI isoform are not due to differences in protein level expression as Western blot analysis (data not shown) and gel shift analysis indicate similar NFI protein levels in transfected cells (Fig. 1C, lanes 3–6, arrow B), which are appropriately super-shifted when incubated with the anti-HA antibody (lanes 8–11, arrow A).

NFI-mediated activation of pNFIβ-gal is dependent on the upstream NFI-binding site as pDNFIβ-gal which lacks an upstream NFI-binding site, is not activated by any of the NFI isoforms (Fig. 2A, lanes 7–10 versus 6). Interestingly, pNFIβ-gal has an ~2-fold higher basal activity than pΔNFIβ-gal (Fig. 2A, lanes 6–10 versus 11–15).
Promoter-specific Activation by NFI

FIG. 2. Promoter-specific activation by NFI requires an NFI-binding site, glucocorticoid receptor, and COOH-terminal activation domains. Panel A, JEG-3 cells were transfected with 5 μg of either pNFIβ-gal (lanes 1–5) or pΔNFIβ-gal (lanes 6–10), and co-transfected with 2.5 μg of a CMV control vector (lanes 1 and 6), or vectors expressing NFI-A (lanes 2 and 7), NFI-B (lanes 3 and 8), NFI-C (lanes 4 and 9), NFI-X (lanes 5 and 10), and 2.5 μg of the SV-40 luciferase internal control vector. β-Gal expression was normalized to luciferase values with the bars representing the mean and range of four measurements from duplicate transfections. Panel B, reporter pMMTVβ-gal (5 μg) was transfected alone (lanes 1–10), or with 1.0 μg of a hGR expression vector (lanes 11–20), and co-transfected with 2.5 μg of CMV control vector (lanes 1, 2, 11, and 12), CMV vectors expressing NFI-A (lanes 3, 4, 13, and 14), NFI-B (lanes 5, 6, 15, and 16), NFI-C (lanes 7, 8, 17, and 18), or NFI-X (lanes 9, 10, 19, and 20) into JEG-3 cells which were cultured in the absence or presence of 0.1 μM dexamethasone (+ or − Dex) for 24 h. β-Galactosidase activity was normalized to luciferase levels and is expressed as fold activation over the CMV control plasmid (lanes 1, 2, 11, and 12). Panel C, reporter plasmid pMMTVβ-gal (5 μg) and a hGR expression plasmid (1 μg), or pNFIβ-gal (5 μg), were transfected with 2.5 μg of either CMV control vector (lanes 1, 2, and 11) or vectors expressing full-length NFI-B (lanes 3 and 4), NFI-X (lane 12), or the truncated DNA-binding domains (see “Experimental Procedures” for construction) of NFI-B (lanes 5, 6, and 13), NFI-C (lanes 7, 8, and 14), or NFI-X (lanes 9, 10, and 15). β-Gal activity was normalized to luciferase levels and is expressed as fold activation over the CMV control plasmid.
These findings demonstrate that the promoter-specific differences in relative activation potential of NFI-B and NFI-X appear to be mediated solely by their COOH-terminal regions.

Differences in Activation Potential of NFI-B and -X Are Not Due Solely to the Presence of GREs—While both reporter constructs used in this study are NFI-dependent, the MMTV promoter is considerably more complex, with its activity also dependent on GREs and other sequence motifs (reviewed in Ref. 45). To determine if the promoter-specific differences in relative activation potentials of the NFI-X and NFI-B isoforms were due to the presence of GREs, we compared the activation properties of pNFI\(_{b}\)-gal with those of pNFIGRE\(_{b}\)-gal, which was made by addition of two GREs upstream of the NFI-binding site of pNFI\(_{b}\)-gal (Fig. 5A). In the absence of coexpressed GR, the activity of pNFIGRE\(_{b}\)-gal appears identical to that of pNFI\(_{b}\)-gal with NFI-B activating 10-fold and NFI-X 15-fold (Fig. 5B, lanes 7–12 versus 1–6, see also Fig. 2A). The lack of dexamethasone induction here is due to the absence of either GREs (lanes 2, 4, and 6) or GR (lanes 8, 10, and 12). In the absence of dexamethasone, when GR is coexpressed with pNFI-GRE\(_{b}\)-gal, the reporter expression is identical to that seen with pNFI\(_{b}\)-gal with NFI-B and NFI-X activating expression ~10-fold and ~15-fold, respectively, (Fig. 5B, lanes 13 and 17 versus 13). However, a robust dexamethasone-dependent activation occurs when the human glucocorticoid receptor is coexpressed with pNFIGRE\(_{b}\)-gal (~150-fold, lanes 14 versus 13). Dexamethasone-dependent activation is further enhanced when NFI-B is co-transfected (lane 16, ~260-fold).

### FIG. 3.
All 4 NFI proteins are present in the cell nucleus. Vectors expressing each of the HA-tagged NFI proteins were transfected into HeLa cells, the cells were fixed and stained with anti-HA monoclonal antibodies and fluorescein isothiocyanate-conjugated antisera and the cells were examined by fluorescent microscopy. The panels show cells transfected with vectors expressing NFI-A (panel A), NFI-B (panel B), NFI-C (panel C), NFI-X (panel D), or empty CMV vector (panel E). The white outlines in panel D indicate the approximate cell borders seen in much longer exposures of the same field and illustrate the extent of nuclear localization.

### FIG. 4.
Relative activation potentials of NFI-B and NFI-X are mediated by their COOH-terminal regions. JEG-3 cells were transfected with 5 μg of either pMMTV\(_{b}\)-gal and a hGR (1 μg) expression vector (lanes 1–10) or pNFI\(_{b}\)-gal (lanes 11–15) and co-transfected with 2.5 μg of an empty CMV plasmid (control, lanes 1, 2, and 11), or vectors expressing NFI-B (lanes 3, 4, and 12), NFI-X (lanes 7, 8, and 14), NFI-BX (lanes 9, 10, and 15), or NFI-XB (lanes 5, 6, and 13). Cells transfected with pMMTV\(_{b}\)-gal were cultured in the absence or presence of 0.1 μM dexamethasone for 24 h (~or + Dex). β-Gal activity was normalized to luciferase and is plotted as in Fig. 2.

NFI-X/B (Fig. 4, lanes 14 and 15 versus 12 and 13). These findings demonstrate that the promoter-specific differences in relative activation potential of NFI-B and NFI-X appear to be mediated solely by their COOH-terminal regions.

### FIG. 5.
Differences in promoter-specific activation potential are not due solely to GREs. Panel A, schematic representation of pNFIGRE\(_{b}\)-gal created by cloning GREs (closed hatched ovals) upstream of the NFI-binding site (closed circle) in pNFI\(_{b}\)-gal as described under “Experimental Procedures.” Panel B, JEG-3 cells were transfected with either 5 μg of pNFI\(_{b}\)-gal and 1 μg of phGR (lanes 1–6), 5 μg of pNFIGRE\(_{b}\)-gal (lanes 7–12), or 5 μg of pNFIGRE\(_{b}\)-gal and 1 μg of phGR (lanes 13–18), and co-transfected with 2.5 μg of the CMV expression vectors indicated below the figure. β-Gal activity of extracts prepared from cells cultured in the absence or presence of 0.1 μM dexamethasone (~or + Dex) for 24 h, is plotted as in Fig. 2.
lated in vitro, to form heterodimers (22), however, the transcription modulation properties of heterodimers were not assessed. To examine the transcription modulation properties of NFI heterodimers, we coexpressed two NFI isoforms in JEG-3 cells. A constant amount of the NFI-X effector plasmid (1.0 µg) and increasing amounts (1.0–5.0 µg) of the NFI-C effector plasmid were co-transfected with pNFIβ-gal (Fig. 6A). Alternatively, 1.0 µg of the NFI-B effector plasmid was co-transfected with increasing amounts (1.0–5.0 µg) of the NFI-C effector plasmid and pMMTVβ-gal (Fig. 6B). As observed previously, NFI-C activates pNFIβ-gal ∼9–11-fold, while NFI-X maximally activates ∼22-fold (Fig. 6A, lanes 2–4 versus 5). Co-transfection of equal amounts of NFI-X and NFI-C effector constructs results in intermediate activation levels, which are the average of the activation mediated by NFI-X and NFI-C homodimers (lane 6 (∼15-fold) versus 5 (∼22-fold) and 2 (∼9-fold)). As the amount of NFI-C effector plasmid co-transfected with the NFI-X effector increases, activation levels approach those observed with NFI-C alone (lane 7 (∼12-fold) versus 3 (∼10-fold) and lane 8 (∼11-fold) versus 4 (∼11-fold)). Similar results are observed in co-transfections with pMMTVβ-gal, where NFI-C activates pMMTVβ-gal ∼7–8-fold upon treatment with dexamethasone (Fig. 6B, lanes 4, 6, and 8) and NFI-B activates ∼16-fold (lane 10), which is reduced to an intermediate level when equal amounts of NFI-B and NFI-C effector plasmids are coexpressed (lane 12 (∼11.5-fold) versus 10 (∼16-fold) and 4 (∼7-fold)). As the amount of NFI-C effector plasmid co-transfected with NFI-B increases, promoter activity approaches activation levels observed with NFI-C alone (lane 14 (∼10-fold) versus 6 (∼8-fold) and lane 16 (∼8-fold) versus 8 (∼8-fold)). These findings indicate that NFI-X/C and NFI-B/C heterodimers exhibit intermediate activation potentials, which appear to be the average of levels observed for homodimers (see “Discussion”).

The VP16 Acidic Domain Is a More Potent Activator Than the NFI Activation Domains—To determine the relative potencies of the NFI activation domains, we compared the activation properties of NFI domains to that of the potent VP16 acidic activation domain (46). For this comparison, the COOH-terminal 78 residues of VP16 were fused to the DNA-binding domain of NFI-X (NH2-terminal 210 amino acids) creating effector plasmid pNFI-X-210-VP16. Co-transfecting 2.5 µg of pNFI-X-210-VP16 with each of our model NFI-dependent reporter constructs, results in a strong activation of each reporter (Fig. 7, lane 10 versus 2 and lane 15 versus 11). If more than 2.5 µg of pNFI-X-210-VP16 plasmid was transfected, a decrease in β-gal expression was seen (data not shown). This decrease is probably due to squelching by the strong transactivation domain of VP16 (47), which is not seen with any of the NFI activation domains (Fig. 2B and data not shown). Activation is dependent on the VP16 activation domain as control plasmid pNFI-X-210, which contains only the NFI-X DNA-binding domain fails to significantly activate either NFI-dependent reporter construct (Fig. 7, lane 4 versus 2 and lane 12 versus 11). VP16-mediated activation is significantly stronger than NFI-X or NFI-B-mediated activation of each NFI-dependent reporter construct (lane 10 versus 6 and 8 and lanes 15 versus 14 and 13), showing that the maximum activity of each promoter is greater than that seen with the most potent NFI activation domain used.

**DISCUSSION**

In this study, we used two model NFI-dependent promoters to assess the transcription activation properties of murine NFI proteins from each of the four mouse genes (NFI-A, -B, -C, and -X). Transfection experiments in JEG-3 cells demonstrate promoter-specific differences in activation potentials for the NFI isoforms, with NFI-X being the most potent activator of a
Promoter-specific Activation by NFI

The VP16 acidic activation domain is more potent than the most potent NFI activation domain. JEG-3 cells were transfected with either 5 μg of pMMTVβ-gal and 1 μg of phGR (lanes 1–10) or 5 μg of pNFIβ-gal (lanes 11–15) and with 2.5 μg of CMV control vector (lanes 1, 2, and 11), NFI-X-210 (lanes 3, 4, and 12), NFI-B (lanes 5, 6, and 13), NFI-X (lanes 7, 8, and 14), or the chimeric NFI-X-210-VPI6 expression vector (lanes 9, 10, and 15). Cells were cultured for 24 h with or without dexamethasone (− or + Dex). Normalized β-gal activity is plotted as in Fig. 2, with the bars representing the mean and range of four measurements from duplicate transfections.

The molecular basis for differential activation by the four NFI isoforms to assess the transactivation potentials of heterodimers, suggests that activation potentials of heterodimers are the average of the activation seen with homodimers, indicating additional mechanisms by which coexpression of different NFI proteins may regulate gene expression (Fig. 6).

While previous studies have suggested that NFI proteins regulate the tissue-specific expression of NFI-responsive genes, little is known about the ability of NFI proteins to modulate transcription in a promoter-specific manner. Human NFI-X2 and CTF1 were both shown to be potent activators when bound to either promoter or enhancer positions on the human papilloma type 16 virus (51). In contrast, human NFI-X1 transactivated only at the promoter position (51), suggesting that NFI-binding site location could mediate promoter-specific differences in the activation potential of these isoforms. Also, Krebs et al. (52) have reported that murine NFI-A1.1 is a more potent activator of the neurotropic JC viral promoter than human CTF1, which has been shown to be a strong activator of other NFI-dependent promoters (13). However, the relative activation potentials of NFI-A1.1 and CTF1 have not been directly compared on other NFI-responsive promoters so it is unknown if these two isoforms exhibit promoter-specific differences in activation potential.

Here we report direct evidence of promoter-specific differences in NFI-dependent activation, with NFI-X mediating the strongest activation of pNFIβ-gal, and NFI-B maximally activating pMMTVβ-gal (Fig. 1B, i versus ii and Fig. 2, A versus B). Promoter-specific differences in activation potential are mediated by their COOH-terminal domains, as swapping these regions transpose their relative activation potentials (Fig. 4). NFI-X remains the strongest activator of the simple promoter which has been modified by the addition of GREs, indicating that the promoter-specific differences in relative activation between the NFI-B and NFI-X isoforms, are not due solely to the presence of GREs (Fig. 5). Coexpression of multiple NFI isoforms to assess the transactivation potentials of heterodimers, suggests that activation potentials of heterodimers are the average of the activation seen with homodimers, indicating additional mechanisms by which coexpression of different NFI proteins may regulate gene expression (Fig. 6).

Previous studies indicated that NFI proteins possess a two-domain structure consisting of highly homologous NH2-terminal DNA-binding domains and more divergent COOH-terminal domains (16, 18, 19, 48). The different maximal activation potentials of the NFI isoforms used in this study appear to be mediated by the COOH-terminal domains, as deletion constructs containing only the DNA-binding domain fail to significantly activate either promoter (Fig. 2C). These findings support previous studies that suggest the transcription modulation properties of the human NFI-C and NFI-X isoforms reside in their COOH-terminal domains (18, 36).

The molecular basis for differential activation by the four NFI proteins studied here is unknown. Previous studies showed that one human NFI-C isoform (CTF1) contains a region homologous to the COOH-terminal domain of RNA polymerase II (CTD), which can physically interact with TBP (33, 49), TFIIB (32), and TAFII55 (50) and is critical for CTF1-mediated activation in yeast (35). However, the NFI-B and -X isoforms used here lack a CTD-like repeat, yet they are potent activators. Also, splice variants of NFI-C proteins, CTF5 and CTF7, which have stronger maximal activation potentials in yeast than CTF1, lack the CTD-like repeat (31, 34). These data indicate that additional unknown protein domain(s) on the NFI proteins can substitute for the CTD repeat in promoting interactions with components of the transcription apparatus and that NFI gene products may differ in the molecular basis of their transcriptional activation. It has also been proposed, that the activation potential of NFI-C isoforms is determined by the proportion of proline residues in the activation domain (18). Our data indicate that the relative composition of proline residues is not the sole determinant of activation potential, as NFI-X with only 9% (17/192 residues) proline in its COOH-terminal domain, is a strong activator of both NFI-dependent promoters. In contrast, the NFI-C isoform has a higher proline content, 13% (30/229 residues), yet has a -2 fold lower maximal activation potential (Fig. 1B, i and ii). Further studies are needed to determine the mechanism(s) by which NFI proteins modulate transcription and to identify potential NFI isoform-specific coactivator proteins.

Promoter-specific differences in NFI-dependent activation, with NFI-X mediating the strongest activation of pNFIβ-gal, and NFI-B maximally activating pMMTVβ-gal (Fig. 1B, i versus ii and Fig. 2, A versus B). Promoter-specific differences in activation potential are mediated by their COOH-terminal domains, as swapping these regions transpose their relative activation potentials (Fig. 4). NFI-X remains the strongest activator of the simple promoter which has been modified by the addition of GREs, indicating that the promoter-specific differences in relative activation between the NFI-B and NFI-X isoforms, are not due solely to the presence of GREs (Fig. 5). Coexpression of multiple NFI isoforms to assess the transactivation potentials of heterodimers, suggests that activation potentials of heterodimers are the average of the activation seen with homodimers, indicating additional mechanisms by which coexpression of different NFI proteins may regulate gene expression (Fig. 6).

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The molecular basis for differential activation by the four NFI proteins studied here is unknown. Previous studies showed that one human NFI-C isoform (CTF1) contains a region homologous to the COOH-terminal domain of RNA polymerase II (CTD), which can physically interact with TBP (33, 49), TFIIB (32), and TAFII55 (50) and is critical for CTF1-mediated activation in yeast (35). However, the NFI-B and -X isoforms used here lack a CTD-like repeat, yet they are potent activators. Also, splice variants of NFI-C proteins, CTF5 and CTF7, which have stronger maximal activation potentials in yeast than CTF1, lack the CTD-like repeat (31, 34). These data indicate that additional unknown protein domain(s) on the NFI proteins can substitute for the CTD repeat in promoting interactions with components of the transcription apparatus and that NFI gene products may differ in the molecular basis of their transcriptional activation. It has also been proposed, that the activation potential of NFI-C isoforms is determined by the proportion of proline residues in the activation domain (18). Our data indicate that the relative composition of proline residues is not the sole determinant of activation potential, as NFI-X with only 9% (17/192 residues) proline in its COOH-terminal domain, is a strong activator of both NFI-dependent promoters. In contrast, the NFI-C isoform has a higher proline content, 13% (30/229 residues), yet has a -2 fold lower maximal activation potential (Fig. 1B, i and ii). Further studies are needed to determine the mechanism(s) by which NFI proteins modulate transcription and to identify potential NFI isoform-specific coactivator proteins.

While previous studies have suggested that NFI proteins regulate the tissue-specific expression of NFI-responsive genes, little is known about the ability of NFI proteins to modulate transcription in a promoter-specific manner. Human NFI-X2 and CTF1 were both shown to be potent activators when bound to either promoter or enhancer positions on the human papilloma type 16 virus (51). In contrast, human NFI-X1 transactivated only at the promoter position (51), suggesting that NFI-binding site location could mediate promoter-specific differences in the activation potential of these isoforms. Also, Krebs et al. (52) have reported that murine NFI-A1.1 is a more potent activator of the neurotropic JC viral promoter than human CTF1, which has been shown to be a strong activator of other NFI-dependent promoters (13). However, the relative activation potentials of NFI-A1.1 and CTF1 have not been directly compared on other NFI-responsive promoters so it is unknown if these two isoforms exhibit promoter-specific differences in activation potential.

Here we report direct evidence of promoter-specific differences in NFI-dependent activation, with NFI-X mediating the strongest activation of pNFIβ-gal, and NFI-B maximally activating pMMTVβ-gal (Fig. 1B, i versus ii and Fig. 2, A versus B). The molecular basis for promoter specificity is unknown, but appears to be mediated by the COOH-terminal regions of the proteins, as swapping these regions transposes relative activation potential (Fig. 4). One possible model to explain these differences may be that NFI isoforms differ in their ability to interact with other transcription factors binding to adjacent sites in the promoter. pMMTVβ-gal differs from pNFIβ-gal by the presence of GREs and Oct-1 recognition sequences flanking the NFI-binding site, which are required for maximal hormone induction (53, 54). The addition of GREs to pNFIβ-gal renders it glucocorticoid responsive, but does not alter the relative activation potentials of the NFI-B and NFI-X proteins, with NFI-X being the strongest activator of both pNFIβ-gal and pMMTVβ-gal (Fig. 5B). These findings suggest that the promoter-specific differences in relative activation potential are not due solely to the presence of GREs and may be determined by additional sequence element(s). In a previous study, adjacent Oct-1 and NFI-binding sites in the human papilloma virus type 16 enhancer were found to activate cooperatively by the ability of Oct-1 to stabilize NFI binding to the enhancer in epithelial cells (55). However, the composition of NFI isoforms binding to the enhancer in these cells was not determined. It will be of interest to determine if the presence of octamer-binding sites and Oct-1 can influence the relative activation potentials of NFI isoforms.

The NFI-dependent promoters used in this study appear to differ in their activation mechanism, as the MMTV promoter is in a repressed state in the absence of dexamethasone even with
co-transfected NFI effector plasmids (Fig. 2B, --Dex lanes). Activation depends on both NFI and GR (Fig. 2B, lanes 1–10, versus 11–20, +Dex), which is consistent with previous reports (43, 44). This requirement for both NFI and GR for MMTV promoter activation may result from the proposed nucleosomal structure of the MMTV promoter. The MMTV-LTR is arranged into phased nucleosomes in cells stably transfected with minichromosomes containing MMTV-driven reporter genes (56). The spatial distribution of the phased nucleosomes allows access of GR to the GREs, but precludes access of NFI to its cognate site (57). The proposed mechanism of activation is a two-step model where binding of the GR upon hormone treatment initiates a chromatin remodeling event which then enables NFI to bind the promoter, resulting in transcriptional activation (reviewed in Ref. 45). This model postulates an ordered requirement of GR and NFI for dexamethasone-dependent induction of the MMTV promoter. In contrast, pNFI\textbeta-gal expression does not appear to be sequentially dependent on both GR and NFI, as either transcription factor alone activates expression, albeit at different levels (Fig. 5B), suggesting that a GR-dependent chromatin remodeling event may not be required for activation of pNFI-GRE-gal. Thus, the potentially different roles for NFI in the activation of pMMTV\textbeta-gal and pNFI\textbeta-gal could account for the promoter-specific differences in relative activation potentials of the NFI-B and NFI-X proteins.

NFI proteins have been shown to form heterodimers both in vitro and in transfected cells (22, 58). Our previous observation that NFI genes are expressed in unique but overlapping patterns during both embryonic development and in adult tissues (24) suggest that NFI heterodimers are also likely to be present in vivo. Our data suggests that heterodimers have intermediate activation potentials which are the average of the activation potentials of homodimers. This conclusion is based on the assumption that under conditions where equal amounts of two NFI isoforms are coexpressed, 25% of the molecules are homodimers of one, 50% are heterodimers, and 25% are homodimers of the other. As the NFI-B, -C, and -X proteins are of similar molecular weights, we were unable to distinguish between homo- and heterodimers by gel mobility shift analysis. However, to test the ratio of heterodimers formed between NFI-C and NFI-B, we coexpressed equal amounts of the full-length NFI-B effector plasmid with the truncated NFI-C-240 effector plasmid, which has DNA binding and dimerization properties similar to the full-length NFI-C protein (data not shown). Under these conditions, the expected distributions of 25% NFI-B homodimers, 50% NFI-B/C-240 heterodimers, and 25% NFI-C-240 homodimers were observed as determined by gel mobility shift analysis (data not shown).

To calculate the transactivation potentials of NFI heterodimers, we used the following model. Transfecting 1 µg of the NFI-C effector plasmid activates pNFI\textbeta-gal ~9-fold, while 1 µg of the NFI-X plasmid results in ~22-fold activation (Fig. 6A). However, when 1 µg each of the NFI-C and NFI-X plasmids are transfected, NFI-C and -X homodimers each comprise 25% of NFI dimers in transfected cells. Their relative contributions to activation can be calculated as: 0.25 (4.5-fold + 4.5-fold) = 2.25-fold for NFI-C and 0.25 (11-fold + 11-fold) = 5.5-fold for NFI-X. NFI-X/NFI-C heterodimers represent ~50% of the NFI population, with their relative activation potentials being: 0.50 (4.5-fold + 11-fold) = 7.75-fold. If this model is correct, we would expect maximal activation to be a sum of the relative contributions of homo- and heterodimers: (2.25-fold + 5.5-fold + 7.75-fold) = 15.5-fold, which is nearly identical to the experimentally observed ~15-fold activation of pNFI\textbeta-gal when 1 µg each of NFI-X and NFI-C effector plasmids are coexpressed in JEG-3 cells (Fig. 6A). Similar intermediate activation levels are observed when 1 µg each of NFI-B and NFI-C are coexpressed in transfections with the MMTV reporter construct (Fig. 6B), suggesting that heterodimers behave similarly on both NFI-dependent promoters. Our data suggesting that heterodimers have activation properties which are the average of homodimers is consistent with a model in which the maximal activation seen is the sum of the activation potentials of each activation domain recruited to the promoter. These findings suggest that when dimeric NFI binds to a promoter element, two copies of an activation domain are required for maximal activation and that a single copy of the activation domain leads to ~50% of maximal activation. In contrast, if a single copy of an activation domain present at a promoter could maximally activate and if different NFI activation domains function through independent mechanisms to activate transcription, then heterodimers containing one copy of each activation domain would have a greater maximal activation potential than the most potent homodimer. Clearly this is not the case with heterodimers of the NFI isoforms used here. It will be of interest to determine if NFI heterodimers modulate transcription in a similar manner on other NFI-dependent promoters.

It is important to note that the four NFI isoforms tested in this study represent only a small subset of the known differentially spliced isoforms of NFI and that we have only tested two NFI-dependent promoters in this study. It is therefore likely that the differences in maximum activation potential, and promoter specific differences in activation potential reported here, do not reflect the maximal difference between the transcription modulation properties of all NFI isoforms. Also, the stronger activation observed with VP16 (Fig. 7), suggests that the maximal activation values observed with the various NFI isoforms may not necessarily represent the maximal NFI-mediated activation of all isoforms. As the JEG-3 cells used in this study provide a near null background, it would be of particular interest to test the ability of various NFI isoforms to activate or repress the expanding number of tissue-specific and developmentally-regulated NFI-dependent promoters. The data presented here indicate that both homo- and heterodimers of NFI isoforms would likely exhibit both promoter-specific, and potentially cell-type specific modulation of such developmentally regulated promoters.

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