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# Selective Regulation of Gene Expression by Nuclear Factor 110, a Member of the NF90 Family of Double-stranded RNA-binding Proteins

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Members of the nuclear factor 90 (NF90) family of double-stranded RNA (dsRNA)-binding proteins have been implicated in several biological processes including the regulation of gene expression. cDNA sequences predict that the proteins have a functional nuclear localization signal and two dsRNA-binding motifs (dsRBMs), and are identical at their N termini. Isoforms are predicted to diverge at their C termini as well as by the insertion of four amino acid residues (NVKQ) between the two dsRBMs. In this study, we verified the expression of four of the isoforms by cDNA cloning and mass spectrometric analysis of proteins isolated from human cells. Cell fractionation studies showed that NF90 and its heteromeric partner, NF45, are predominantly nuclear and largely chromatin-associated. The C-terminally extended NF90 species, NF110, are almost exclusively chromatin-bound. Both NF110 isoforms are more active than NF90 isoforms in stimulating transcription from the proliferating cell nuclear antigen reporter in a transient expression system. NF110b, which carries the NVKQ insert, was identified as the strongest activator. It stimulated transcription of some, but not all, promoters in a fashion that suggested that it functions in concert with other transcription factors. Finally, we demonstrate that NF110b associates with the dsRBM-containing transcriptional co-activator, RNA helicase A, independently of RNA binding.

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*Keywords:* NF90; NF110; DRBP76; ILF3; NFAR

## Introduction

Many aspects of gene expression are controlled by proteins that bind to structured elements in RNA molecules. Double-stranded RNA (dsRNA) and related duplex structures are recognized by a protein domain known as the dsRNA-binding motif (dsRBM), of 65–70 amino acid residues.[1] This motif binds dsRNAs and highly structured single-stranded RNA molecules, with a lesser affinity for RNA/DNA hybrids and unstructured single-stranded RNAs, and mediates protein–protein interactions between dsRBM-containing proteins. The dsRBM is found in an expanding family of proteins involved in the regulation of gene expression at several levels, including mRNA transcription, splicing, editing and export, RNA interference, and translation.[2] Despite the identification of an estimated 26 dsRBM proteins in the human genome, the functions of several of these proteins have yet to be fully defined.

One such group of proteins is the nuclear factor 90 (NF90) protein family (Table 1), members of which contain two dsRBMs. The founding human member of this family, NF90, was isolated together with its partner protein, nuclear factor 45 (NF45),

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Abbreviations used: NF, nuclear factor; dsRNA, double-stranded RNA; dsRBM, dsRNA-binding motif; ILF3, interleukin enhancer-binding factor 3; RHA, RNA helicase A; PCNA, proliferating cell nuclear antigen; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; ECL, enhanced chemiluminescence.

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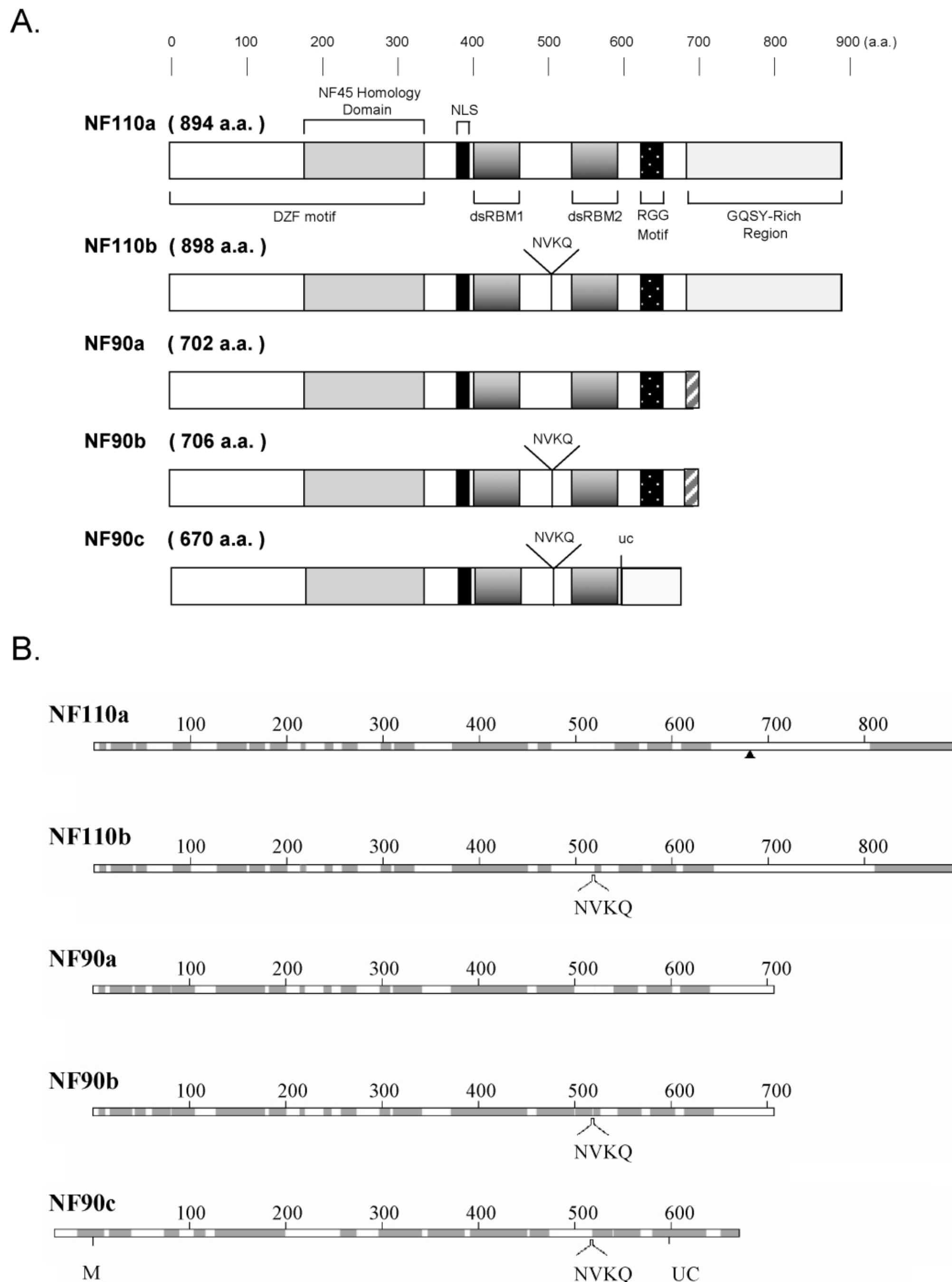


**Figure 1** A and B (*legend opposite*)

as a DNA-binding transcription factor specific for NF-AT sites.[3,4] NF45 is derived from a different gene locus, but contains a region of approximately 150 amino acid residues that is homologous to NF90 family members (Figure 1A). Subsequently, a C-terminally extended isoform of NF90, here called NF110, was detected by immunological techniques and cDNA cloning.[5–9] *Xenopus* homologues of NF90 and NF110 were independently cloned and characterized as dsRNA-binding proteins.[10–12] Several further members of the protein family have been identified by a variety of routes.[6,8,13–15] More recently, it was determined that many of the human homologues are generated through the alternate splicing of transcripts from the interleukin enhancer-binding factor 3 (ILF3) gene locus.[5,7]

As a result of their associations with cellular proteins and complexes, NF90 and its homologues have been implicated in a variety of cellular processes. Several lines of evidence suggest a role for these proteins in translation regulation. NF90 and its homologue TCP80 were shown to inhibit the translation of several mRNA species *in vitro*.[14,16] Furthermore, NF90 and its homologues interact

C. **884.43 Da**
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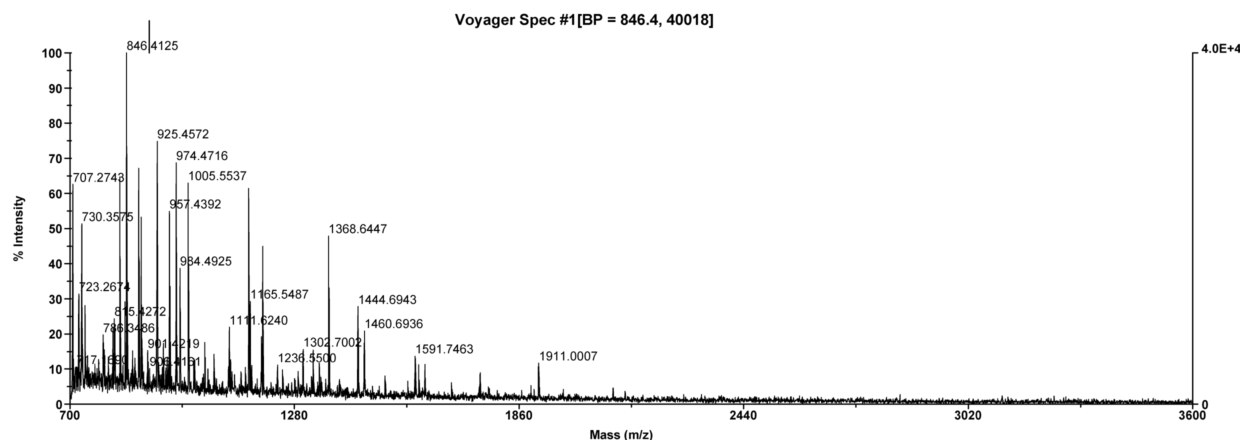


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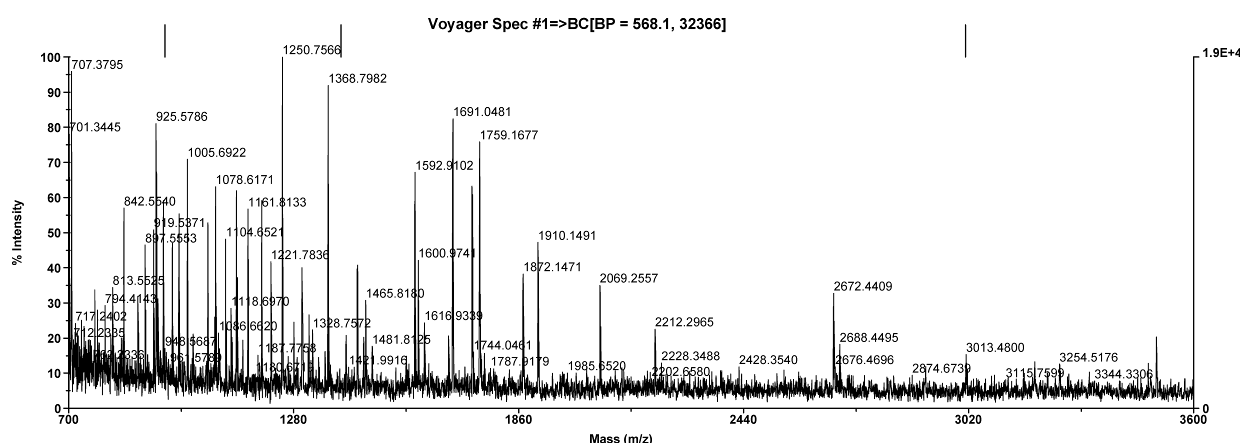


**Figure 1**. Isoforms of NF90 and NF110. A, Proteins predicted from cDNAs corresponding to NF90 family members. B, Amino acid sequence determination of NF90 and NF110 variants by mass spectrometry. HeLa cell 110 kDa and 90 kDa proteins were digested with trypsin and analyzed on a Voyager DE-Pro MALDI-TOF mass spectrometer (Micromass, Manchester, UK). Subsequently, the same proteins were subjected to dual digestion with trypsin and V8 proteinase and analyzed by Micromass UK (Manchester, UK) on a QTOF2 tandem mass spectrometer, using ProteinLynx software. Peptide mass data from the two experiments were combined. Shaded areas represent sequence coverage for the NF110a, NF110b, NF90a and NF90b splice variants. The position of the tetrapeptide insert, NVKQ, is shown and the C-terminal divergence point of the splice variants is indicated by an arrowhead. Tryptic peptides from epitope-tagged NF90c were analyzed by MALDI-TOF mass spectrometry and the resultant coverage map is displayed below. *M* marks the position of the first NF90c methionine residue, which is preceded by 39 amino acid residues of epitope tag. The position on the amino acid sequence of the inserted dinucleotide is marked *UC*. (C) Mass spectra of NF110 tryptic peptides after elution from a C18 ZipTip in 20% (top) and 60% (bottom) acetonitrile. Both spectra were generated on a Voyager DE-Pro MALDI-TOF mass spectrometer in positive ion (M + H) reflector mode. Peptides unique to the NF110 C terminus, and a single peptide of mass 884.43 specific to the b isoforms, are highlighted.

with and serve as substrates for the dsRNA-activated protein kinase PKR, an important cellular regulator of translation.[6,13,17–19] NF90 family members have been purified from human tissue in a complex with both the protein synthesis initiation factor eIF2 and the kinase DNA-PK, suggestive of a role in DNA repair.[20] The interaction of NF110 with the putative splicing factor, TLS, and its interaction with a cellular spliceosome complex has led to the proposal that it plays a role in splicing.[6] The M-phase specific phosphorylation of NF90[15] and its over-expression in cancer cells[21] have also implicated the protein in the regulation of cell-cycle progression.

Strong evidence pointing to a transcriptional role comes from *in vitro* transcription assays and transient expression assays showing that NF90 can function as both a positive and negative regulator of gene expression.[6,22,23] Both NF90 and NF45 were found to be essential for activated transcription of an ARRE-2-driven reporter *in vitro*.[3] Furthermore, NF45 enhanced the activity of NF90 as a transcriptional activator in transient expression assays.[22] The stable expression of NF90 in human

**Table 1.** NF90 homologues

| Species | NF90a/b | NF110a/b | Unique cDNAs |
|---|---|---|---|
| *Homo sapiens* | DRBP76a<br>DRBP76b<br>NFAR 1 | ILF3a<br>ILF3b<br>NFAR2 | NF90c<br>TCP80<br>DRBP76α<br>DRBP76δ |
| *Mus musculus* | NI | ILF3 | |
| *Rattus norvegicus* | NI | ILF3 | |
| *Xenopus laevis* | 4F.1, CBTF[98] | 4F.2, CBTF[122] | |

NI, cDNA not identified. The a or b suffix indicates the absence or the presence of the NVKQ insertion, respectively. For references, see the text.

osteosarcoma cells led to the transcriptional induction of a large group of interferon-responsive genes and resistance to HIV-1 infection,[23] suggestive of a role in the antiviral response. In our laboratory, NF90 was first detected as a dsRNA-binding protein that interacts with dsRNA and adenovirus VA $RNA_{II}$,[24] a relative of the viral VA $RNA_{I}$ species that neutralizes the interferon-induced antiviral response mediated by PKR.[25] The binding of NF90 to a site in the HLA-DRα promoter correlates with down-regulation of the gene.[26] Furthermore, the *Xenopus* proteins CCAAT box transcription factor CBTF[98] and CBTF[122] (first isolated as 4F.1 and 4F.2) are components of a complex that binds to the CAATT box enhancer element present in the GATA-2 promoter.[11,12] The subcellular localization of the proteins and presumably their activity is regulated by interactions with cellular RNA.[12,27] In *Xenopus*, these proteins move from the cytoplasm to the nucleus at the midblastula transition when cytoplasmic RNA is turned over or in response to RNase injection.[12] The movement of the CBTF complex to the nucleus and its binding to the enhancer element is thought to coincide with activation of the developmentally regulated GATA-2 gene, implying a role for NF90 family members in gene expression and embryonic development.

To explore the transcriptional properties of the human members of this protein family, we identified isoforms of NF90 and NF110 by cloning and mass spectrometric analysis, and compared their ability to activate gene expression. We found that both NF90 and NF110 are chromatin-bound, and that NF110 is associated particularly tightly. In a transient reporter assay, both proteins activated expression from the proliferating cell nuclear antigen (PCNA) promoter. This growth-regulated promoter was previously found to be highly sensitive to NF110b in transient reporter assays.[27] The NF110 isoforms were more active than the NF90 isoforms in this assay. Maximal activity was displayed by NF110b, which contains an insertion of four amino acid residues between its dsRBMs. NF110b activated some but not all cellular and viral promoters, and different reporter genes driven by the same promoter were activated selectively. Deletion analysis of the PCNA promoter showed that NF110b activated transcription when a transcription factor-binding site was present but had little effect on the basal promoter. Finally, we report that NF90 family members interact with the transcriptional co-activator RNA helicase A (RHA) both *in vivo* and *in vitro*. This work establishes that members of the NF90 family differentially enhance gene transcription from several different promoters, and that the response depends on the nature of the target gene.

## Results

### NF90 and NF110 isoforms in human cells

Following the characterization of the original NF90 and 4F clones,[4,10] cDNAs encoding immunologically related proteins with apparent molecular mass of approximately 90 kDa and 110 kDa have been isolated from several vertebrate species, including human, mouse, rat and *Xenopus*, using various methods and assays (Table 1). The putative protein products of these cDNAs are similar in sequence, especially at their N-terminal ends (Figure 1A). Examination of the ILF3 gene revealed that the cDNAs result from alternate splicing, yielding different putative protein C termini.[5,7] Further diversity results from additional alternate splicing events giving rise to a 12 nt exon extension specifying an insert of four amino acid residues (NVKQ) between the two dsRBMs. We refer to the putative isoforms that carry this insert as NF90b and NF110b; the corresponding isoforms lacking the insert are termed NF90a and NF110a.

To characterize the NF90/NF110 isoforms present in human cells at the protein level, we purified the endogenous proteins of 90 kDa and 110 kDa by affinity chromatography and compared them to sequences predicted from cDNA clones. Four cDNAs, corresponding to NF90a, NF90b, NF110a, and NF110b (Figure 1A), were isolated by screening a human placental library. No other cDNA was detected, not even that corresponding to the originally isolated NF90 clone (herein referred to as NF90c). The NF90c cDNA contains a 2 bp insertion resulting in synthesis of a protein with a different, slightly shortened C terminus (Figure 1A).

The NF90 and NF110 proteins from HeLa cells were digested and analyzed by mass spectrometry. Peptides identified experimentally were matched with those predicted from the cDNA sequences, and the results are summarized in Figure 1B. We identified peptides corresponding to both NF90 and NF110, confirming the cDNA sequences with a coverage of 62% and 50%, respectively. Peptides containing the NVKQ insertion were identified positively in both the NF90 and NF110 fractions, confirming the existence of the NF90b and NF110b (Figure 1C, top) isoforms. Gaps in coverage

precluded definitive statements about the absence of NVKQ from NF90a and NF110a isoforms. Peptides corresponding to the C-terminal region of the NF110 isoforms were identified (Figure 1C, bottom), but no peptide corresponding to the NF90 C terminus was characterized, probably because of the scarcity of tryptic and GluC cleavage sites in this region. The C-terminal peptide predicted for NF90c was not detected by analysis of the endogenous 90 kDa protein, but it was generated from the 90 kDa protein isolated from a cell line constructed to express this cDNA. We infer that the C terminus of the predominant HeLa cell 90 kDa proteins is probably not that of NF90c, although it has not been identified positively. With this as a limitation, these data indicate that NF90a, NF90b, NF110a, and NF110b are the principal members of the NF90 family present in human cells.

## NF90 and NF110, together with NF45, are associated with chromatin

NF90 and related proteins have been found to localize in the nucleus by immunofluorescence of fixed cells and EGFP epitope-tagging in living cells.[4,6,13,15,22,28] When examined individually, all five of the isoforms illustrated in Figure 1A were predominantly nuclear in 293 cells (data not shown). Strong nuclear localization requires a functional nuclear localization signal just upstream of dsRBM1.[22] A fraction of NF90 is found in the cytoplasm after cell fractionation,[15,17,18,24] and the protein localizes in the cytoplasm during mitosis.[15]

To determine whether the nuclear distribution of NF90 and NF110 is due to their association with chromatin, cells were separated into a cytoplasmic fraction (S2) and nuclear fractions. The nuclei were further separated into chromatin-containing and soluble fractions (P3 and S3, respectively). A sample of the nuclei was digested with micrococcal nuclease prior to fractionation to disrupt chromatin. Analysis of the fractions by gel electrophoresis and Western blotting showed that NF90 and NF110 were predominantly nuclear (~90% and almost 100%, respectively; Figure 2, top panel: compare lane 2 with lanes 3 and 4). A large proportion of the NF90 (~40%) and a majority of the NF110 (~70%) were present in the chromatin-containing nuclear pellet fraction (compare lane 4 with lane 3). Both proteins were released quantitatively by treatment of the nuclei with micrococcal nuclease (lanes 5 and 6). Note that the antibody reacted with both NF90 and NF110, and that the gels did not resolve the a and b forms of the two proteins. Similar results were obtained when the blots were probed using an antibody against NF45 (Figure 2, second panel). To validate the fractionation procedure, blots were probed with antibody against the cytoplasmic protein TRAF2 (third panel) and stained for histones (bottom panel) to monitor the behavior of chromatin. Taken together, these data indicate that NF90, NF110, and NF45, all localize predominantly to the nucleus and are tightly associated with chromatin.

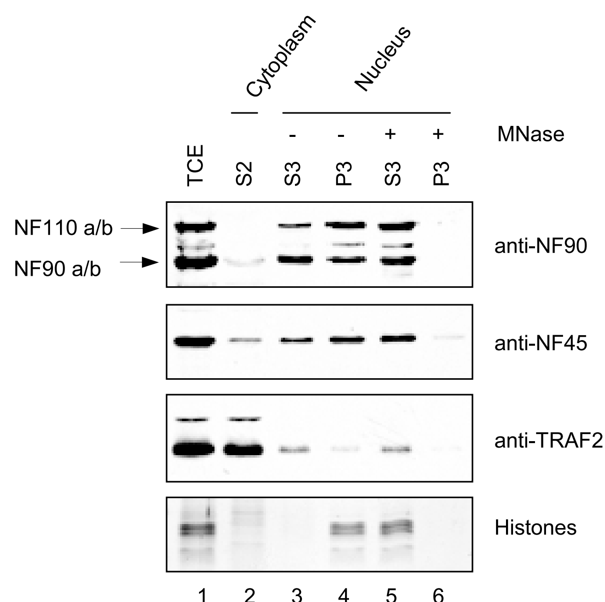


**Figure 2.** Chromatin association of NF90, NF110, and NF45. Jurkat cells were fractionated to give soluble cytoplasmic extract (S2), soluble nuclear extract (S3), and nuclear pellet (P3). Equal volumes of each fraction, and of the total cell extract (TCE), were subjected to gel electrophoresis. Western blots were probed with antibodies directed against NF90, NF45, and TRAF2 (a positive control for the cytoplasmic fraction) and staining by Coomassie brilliant blue to visualize histones (a positive control for the chromatin fraction). The indicated nuclear samples were incubated with micrococcal nuclease (MNase) before fractionation. The a and b isoforms could not be differentiated and are therefore denoted NF90 a/b and NF110 a/b.

## Differential activity of NF90 and NF110 isoforms

NF90 proteins have been reported to regulate gene expression driven by several promoters in transfected mammalian cells.[6,22,23] Both positive[6,22,23] and negative[22,23] effects have been observed. To examine the activities of individual isoforms of NF90 and NF110, we conducted transient expression assays in HeLa cells using the proliferating cell nuclear antigen (PCNA) promoter, which was previously reported to be activated strongly by NF110b.[27] The PCNA-CAT reporter construct was co-transfected with vectors encoding the five isoforms, which differ at their C termini and in the presence or in the absence of the tetrapeptide insert NVKQ between their two dsRBMs (Figure 1A). Each of the proteins stimulated gene expression driven by the PCNA promoter, but to varying extents (Figure 3A, open bars). The order of activity was NF110b > NF110a > NF90a = NF90b = NF90c. Similar results were obtained with epitope-tagged versions of the proteins (Figure 3A, filled bars). Western blots probed with antibody directed against the epitope tag confirmed

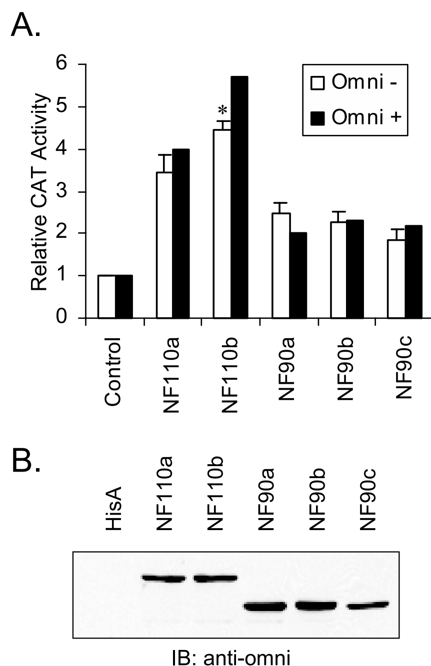


**Figure 3**. Activation of gene expression by NF90 isoforms. A, HeLa cells were transfected with 200 ng of PCNA(-1265)-CAT reporter plasmid and 200 ng of the indicated expression plasmids encoding native proteins (open bars), epitope (omni)-tagged proteins (filled bars), or empty vector control, and 600 ng of HisA control plasmid. After 48 hours, cells were harvested and assayed for CAT activity. Results are plotted as fold activation relative to the control, which was assigned the arbitrary value of 1. Error bars represent the standard deviation ($n = 3$). The asterisk (*) indicates significance at the level of $p < 0.05$ (NF110a *versus* NF110b) obtained using a two-tailed Student's *t*-test. B, Western blot analysis of extract (40 μg) of samples transfected with the epitope-tagged expression plasmid (A, filled bars) probed with omni probe antibody.

that the isoforms were expressed at approximately equivalent levels (Figure 3B). Therefore, the differences in reporter gene expression are principally due to the extended C terminus of the NF110 isoforms, which contains a GQSY-rich region and confers increased activity. The NVKQ insert, present in the b isoforms, makes an additional contribution to this effect when the GQSY-rich region is present (in NF110) but not in its absence (NF90).

## NF110b enhances gene expression from some but not all promoters

To investigate the transcriptional properties of NF110b in detail, we first examined the response of a number of cellular and viral promoters to this protein in transient expression assays. HeLa cells were transfected with a series of reporter constructs together with a vector expressing NF110b, at two concentrations, or a control plasmid. Figure 4A shows that NF110b gave a dose-dependent stimulation of luciferase expression from the PKR and HIV-1 LTR promoters; the magnitude of this effect (about sixfold) was similar to that seen with the PCNA promoter. NF110b had a limited effect on the adenovirus major late minimal promoter (about threefold) and no effect on the ARRE-2-driven IL-2 promoter, which contains NFAT-binding sites.

NF110b also increased expression from the SV40 promoter (Figure 4B). The effect was substantially more pronounced with β-galactosidase as reporter than with firefly luciferase (six- to eightfold *versus* 2.5 to fourfold, respectively). This difference could be due to differential effects on transcription or downstream effects on RNA or protein stability. In view of the greater length of the β-galactosidase mRNA, the difference could be attributable to an action at the level of transcription elongation, a possibility that was raised previously.[6] RNase protection assays were used to determine the levels of β-galactosidase mRNA in the presence and in the absence of NF110b. Figure 4C shows that NF110b elicited an approximately threefold increase in RNA from the reporter gene, consistent with an action of NF110b at the level of transcription. Taken together, these data suggest that NF90 family members, particularly NF110b, enhance the transcription of promoters with some degree of specificity.

## Stimulation by NF110b requires a transcriptionally active promoter

We used deletion mutants of the PCNA promoter to determine whether NF110b acts through a specific enhancer sequence or transcriptional activator. HeLa cells were co-transfected with the PCNA promoter constructs illustrated (Figure 5A) and a plasmid encoding NF110b or its N-terminal truncation (NF110bΔ336N), which gives maximal activity in transient expression assays. As shown in Figure 5B, NF110bΔ336N elicited a fivefold stimulation of expression from the full-length promoter. Removal of *cis*-acting sequences present upstream of the promoter reduced the basal activity of the promoter, but its activity remained sensitive to stimulation by NF110bΔ336 until all upstream transcription sequences were removed. Truncation of the promoter to −46 removes all known transcriptional activator-binding sites apart from an initiator element required for basal transcription.[29] This deletion results in a significant loss in reporter gene activity and in its response to the NF110b mutant. Similar results were obtained using wild-type NF110b (data not shown). Consistent with these data, mutagenesis of the ATF-binding site in the −87 promoter (−87ΔBam) resulted in severely diminished basal activity and loss of NF110b enhancement (Figure 5C). This site is not required specifically for induction by NF110, however. The full-length promoter carrying the mutant ATF site is transcriptionally active,[29] presumably because of the presence of alternate ATF or other transcription factor-binding sites. Since NF110bΔ336N was able to stimulate expression

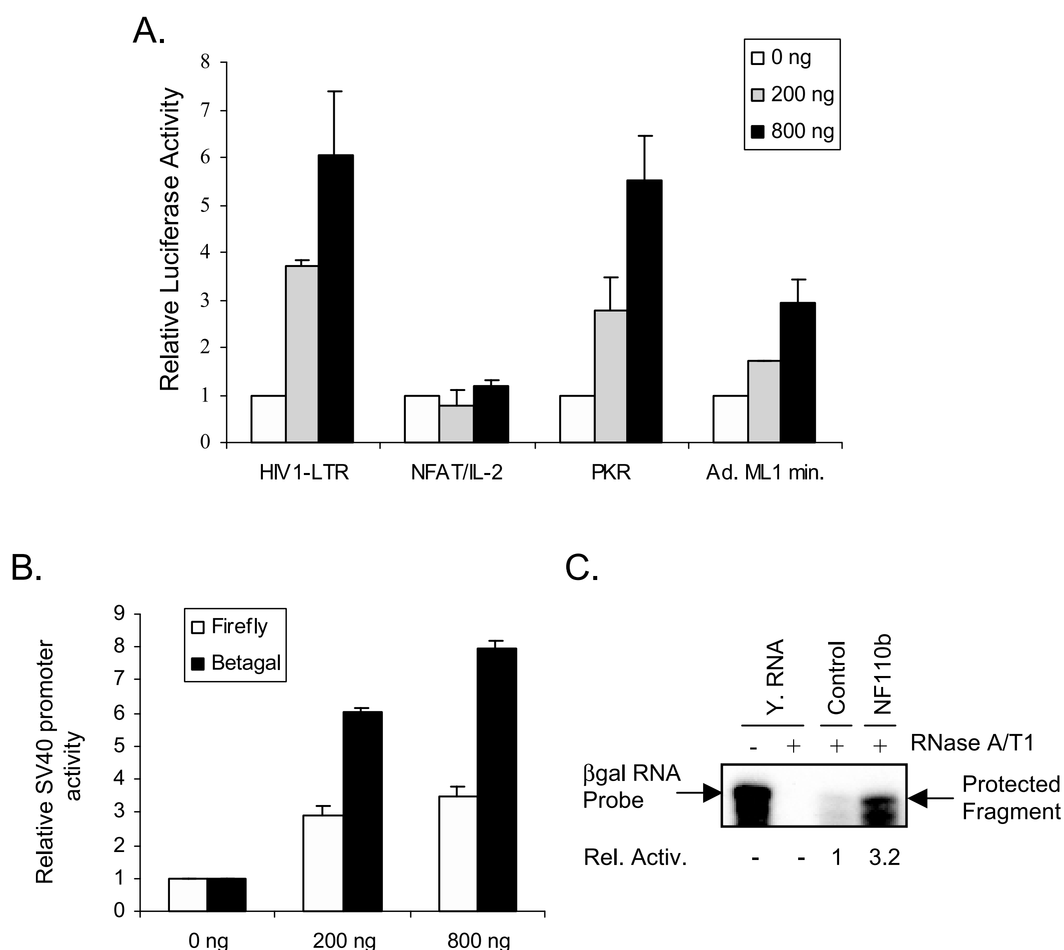


**Figure 4**. Induction of cellular and viral promoters by NF110b. A, HeLa cells were co-transfected with the indicated reporter vector and 0 ng, 200 ng, or 800 ng of NF110b vector (open, gray, and black bars, respectively). Cells were lysed 48 hours after transfection and assayed for luciferase activity. Values were normalized relative to their respective controls lacking NF110b, which were assigned the arbitrary value of 1. Error bars represent standard deviation. Relative basal activities of the HIV-LTR, NFAT/IL-2, PKR, Ad ML1 promoters were 19.2, 1.0, 1.7 and 3.2, respectively. B, HeLa cells were co-transfected with SV40-luciferase (open bars) or SV40-βgal (filled bars) and 0 ng, 200 ng, or 800 ng of NF110b vector. After 48 hours, cells were harvested and assayed for firefly luciferase or β-galactosidase activity. Results are presented as in A. C, Relative levels of β-galactosidase mRNA in transfected HeLa cells (B) were analyzed by RNase protection assay using equal amounts of total cell RNA or yeast RNA (Y. RNA) and an anti-sense β-galactosidase probe. Protected RNA fragments were separated by denaturing gel electrophoresis and quantified using a Packard Instant-imager. Transcript levels are listed relative to the empty vector control, which was assigned the arbitrary value of 1.

about fivefold driven by the −1265ΔBam promoter (Figure 5C), we conclude that the presence of a transcriptional activator or the existence of an activated transcription state is required for NF110b to exert its maximal effect.

### Interaction of NF110b with RNA helicase A

NF90 was found to interact with RHA in a yeast two-hybrid assay (C.-G.L., unpublished results). RHA functions as a transcriptional co-activator at CBP/CREB sites,[30,31] raising the possibility that NF90 might activate transcription in mammalian cells *via* RHA. The interaction between these proteins was confirmed in GST pull-down experiments (Figure 6A). RHA synthesized *in vitro* bound to GST-NF90c but not to GST itself, indicative of a direct interaction. The levels of RHA correlate with those of NF90 in several cell lines.[16] To determine whether the two proteins interact *in vivo*, 293 cell extracts were subjected to immunoprecipitation followed by Western blotting. RHA was detected in complexes immunoprecipitated with anti-NF90 antibody from both nuclear and cytoplasmic extracts, and *vice versa*, but control antibodies (anti-TRAF2 and preimmune serum) gave no signal (Figure 6B). The co-immunoprecipitation of endogenous RHA and NF90 strongly indicated that they interact *in vivo*.

Like the NF90 family proteins, RHA is a dsRNA-binding protein containing two dsRBMs, raising the possibility that complexes between the proteins are bridged by RNA. Although the complexes were still recovered from cell extracts that had been

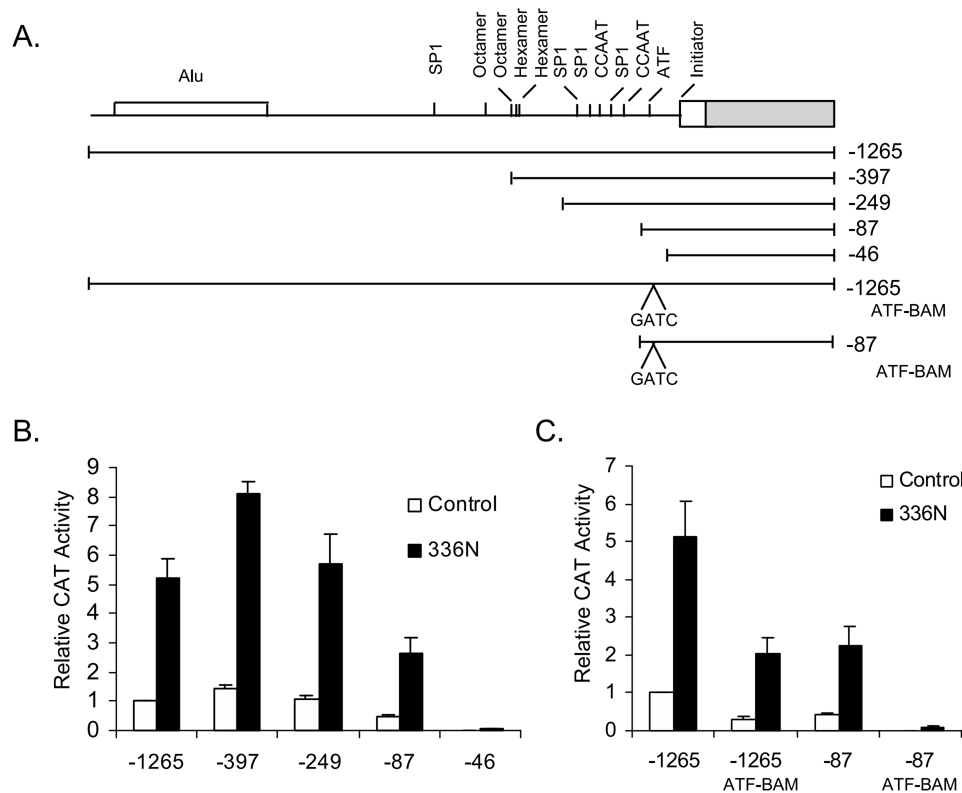


**Figure 5**. Transactivation of PCNA promoter deletion mutants by NF110b. A, A representation of PCNA promoter and PCNA-CAT mutant reporter plasmids. B and C, HeLa cells were transfected with the indicated reporter vector, without (open bars) or with (filled bars) 200 ng of NF110bΔ336N. Cells were lysed 48 hours after transfection and assayed for CAT activity. Assay values were normalized to samples from cells transfected with PCNA-1265 CAT vector and an empty control vector (assigned the arbitrary value of 1). Error bars represent the standard deviation.

treated with RNase A, and were resistant to moderately high concentrations (~800 mM) of salt in the immunoprecipitation experiments (data not shown), deletion mutants of NF90 lacking the dsRNA-binding region failed to interact with RHA (data not shown). Several previous reports have indicated the importance of the dsRBMs in mediating protein–protein as well as protein–RNA interactions.[17,32,33] To specifically examine the requirement for RNA binding, we employed a series of NF110b mutants that are defective to varying degrees in dsRNA binding (Figure 6C). HeLa cells were transfected with plasmids encoding epitope-tagged wild-type or mutant NF110b,[27] and complexes immunoprecipitated from cell lysates with an antibody directed against the epitope tag were analyzed by blotting with an anti-RHA antibody (Figure 6D, top panel) or the epitope tag (lower panel). The results show that the single-point mutations F432A and F559A, which reduce RNA binding to NF110b, and the double mutation F432A,F559A, which profoundly decreases RNA binding (Figure 6C), had little effect on the RHA:NF110b interaction (Figure 6D). Thus the interaction is not dependent on RNA binding. On the other hand, the double mutant A458P,A588P, which is also highly defective in RNA binding, was almost completely deficient in binding RHA. This substitution of two conserved alanine residues with helix-breaking proline residues was previously reported to cause a severe disturbance of dsRBM structure.[34] We conclude that the binding of RHA to NF110b is RNA-independent but involves protein–protein interactions with the dsRBMs of NF110b.

## Discussion

Members of the NF90 family of dsRNA-binding proteins are identical at their N termini but differ significantly at their C termini and at an internal position, presumably as a result of alternate splicing. Several mRNA species and protein isoforms have been identified in the cell lines and tissue types examined. Interestingly, the 90 kDa and 110 kDa forms appear to be concomitantly expressed in cells, albeit at varying ratios. Despite the spectrum of activities with which this protein family has been associated, the individual contributions of the isoforms to cellular functions has not been defined. We have characterized several isoforms of NF90/NF110 expressed in cultured human cells and have explored their functions in transcription. We find that NF110 binds more tightly to chromatin than NF90, and that the NF110 isoforms are more active in transient expression assays than the NF90 isoforms. The

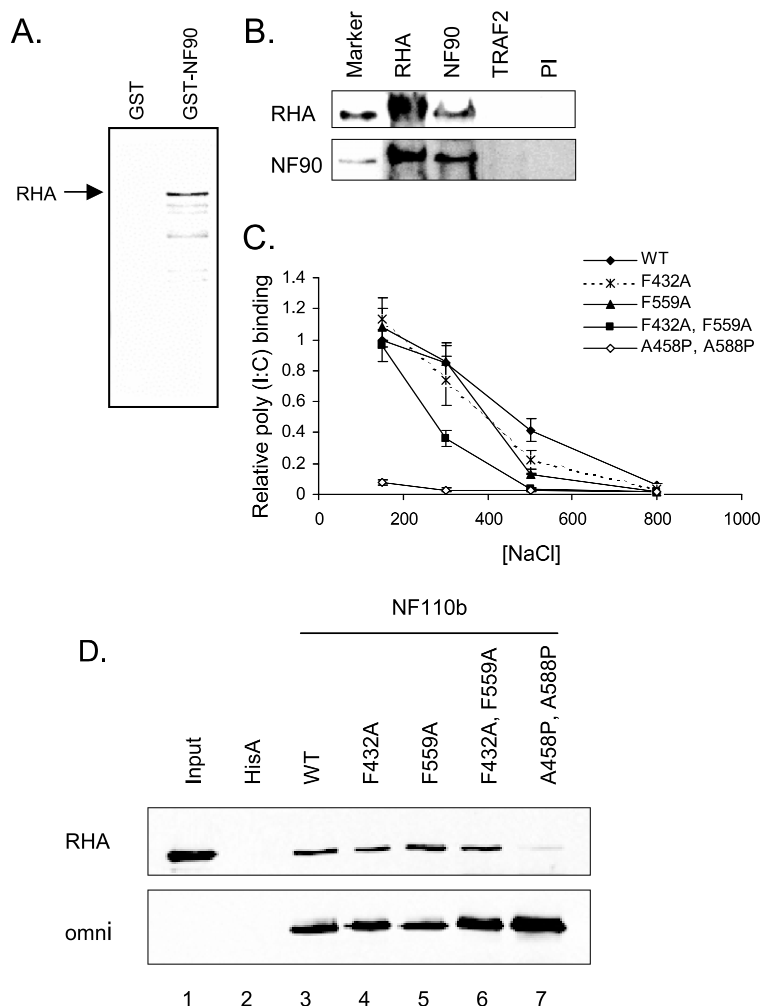


**Figure 6.** Interaction of NF90 family members with RHA. A, Pulldown assays were conducted with $^{35}$S-labeled RHA and beads carrying GST-NF90c or GST alone. Complexes were resolved in SDS-polyacrylamide gels and detected by autoradiography. B, Coimmunoprecipitation assays for RHA (top) and NF91 (bottom) with 293 cell nuclear extracts. Complexes were immunoprecipitated with antibodies against RHA, NF90, or TRAF2, or with preimmune serum, and then blotted with the indicated antibodies. C, Poly(I:C) pulldown of NF110b mutants. Proteins were synthesized in rabbit reticulcyte lysate and incubated with poly(I:C). Samples were washed with increasing concentrations (150–800 mM) of NaCl, and then separated by SDS-PAGE. Gels were then dried and quantified using an Instant Imager (Packard) (D) NF110b coimmunoprecipitates with RHA. Immunoprecipitation reactions were carried out with lysates from HeLa cells transiently transfected with the indicated expression plasmid and the omni probe antibody. Antibody–protein complexes were collected on protein A-Sepharose beads, washed, and separated by SDS-7% PAGE. Proteins were transferred to nitrocellulose and probed with an anti-RHA antibody (1:3000). Membranes were stripped and reprobed with the omni-probe antibody (1:1000).

most active isoform, NF110b, selectively enhances expression driven by some promoters and associates with the transcriptional co-activator RHA.

Compared to NF90, NF110 displays a stronger association with nuclei and chromatin (Figure 2), a greater ability to enhance gene expression from the PCNA promoter (Figure 3), and weaker binding to dsRNA.[27] NF110 was also a stronger inducer of the SV40 promoter than NF90 in Cos[6] and HeLa cells (T.W.R. & M.B.M., unpublished results). These properties correlate with the presence of the GQSY domain at the C terminus of NF110. Like the RGG domain, which is common to all the isoforms tested here except for NF90c, the GQSY domain has the ability to interact with nucleic acids,[35–37] suggesting that it may facilitate the binding of complexes to a promoter. However, since it has proven difficult to demonstrate direct binding of NF90 and its congeners to DNA (see below), the effect of the GQSY domain is more likely to be indirect. Possibly, this domain engages in intramolecular interactions within the protein that weaken its affinity for RNA and/or confer specificity toward particular RNA species. The observation that NF110b is more active than NF110a in gene expression assays, whereas no such difference exists between NF90a and NF90b (Figure 3), may imply that the NVKQ sequence plays a part in modulating these intramolecular interactions.

In HeLa cells, NF110b is a potent activator of the PCNA, PKR and HIV-1 LTR promoters but was unable to stimulate transcription of the ARRE-2-driven IL-2 promoter (Figures 3–5). These findings are consistent with previous observations of promoter specificity with NF90c, which was shown to stimulate transcription directed by the CMV promoter but had little effect on an RSV-driven reporter.[22] Results of the PCNA promoter analysis failed to pinpoint a specific sequence required for activation by NF110b, although promoter activity was evidently a prerequisite and the ATF site could be one such sequence (Figure 5). NF90 and NF110 are present in nuclei in association with chromatin (Figure 2), and electrophoretic mobility-shift assays conducted with their *Xenopus* counterparts revealed weak competition by dsDNA for binding to dsRNA.[10] NF90 lacks discernible sequence-specific DNA-binding motifs, however,

and we have failed to detect direct binding of the human proteins to DNA in either electrophoretic mobility-shift assays or pull-down assays (G. Deyu, L. Parker & M.B.M., unpublished results). Ting *et al*. observed that NF90 was able to cause a DNA supershift only in the presence of DNA-PK and Ku.[20] Taken together, these data suggest that NF90 family members interact with DNA indirectly, by associating with components that themselves bind DNA directly. On the basis of the results shown here, we propose that NF110b activates transcription by interacting with factors bound to the enhancer region of the PCNA promoter but absent from some other promoters. Deletion of these factor-binding sites by promoter truncation or mutagenesis led to decreased activation of the promoter (Figure 5). The presence of additional co-factors (possibly tissue, cell type, or cell stage-specific) appears to regulate the association of NF90/NF110 with several different promoters and dictates selectivity. Strikingly, the SV40 promoter was refractory to NF90 in immortalized mouse embryo fibroblasts, although PKR−/− mouse fibroblasts were sensitive and NF90 was a stronger inducer than NF110.[6]

While several lines of evidence suggest that additional co-factors are required to mediate NF90 function, the nature of the co-factor(s) remains elusive. Analysis of effects on the PCNA promoter (Figure 5) indicates that ATF/CREB could be one such factor. This idea is supported by the interaction of NF90 with RHA, which is likely to be a direct protein–protein interaction (Figure 6). RHA belongs to the DExH box family of RNA helicases[38] and has been implicated in the regulation of transcription *via* the CREB and the mineralocorticoid receptor pathways.[30,31,39] The *Drosophila* homologue of RHA, *Maleless*, is required for transcriptional upregulation of genes on the X chromosome.[40] There are several interesting parallels between these two transcriptional activators. Transcriptional stimulation by NF110 depends on its ability to bind RNA. Mutations in the dsRBMs of NF110b reduced its dsRNA-binding activity and concomitantly decreased the ability of the protein to activate gene expression.[27] Correspondingly, activation by NF110 is tempered by the expression of a non-specific structured RNA.[27] Similarly, dosage compensation, in which *Maleless* participates, also requires the *Drosophila* RNAs *RoX1* and *RoX2*.[41] Furthermore, RHA forms a complex with topoisomerase II that requires RNA.[42] NF110 has been reported to associate with two other helicases, p68 and p72,[43] which play important roles in transcription[44,45] and mRNA splicing.[46,47] Interestingly, NF90 interactions with RHA are RNA-independent (Figure 5D), and it binds to PKR in both RNA-dependent and RNA-independent modes.[17]

Precedents exist for promoter-specific and transcription factor-specific modulation of transcription by RNA-binding proteins,[31,48] and the interaction of the *Xenopus* CBTF with RNA:DNA hybrids[10] raises the possibility that nascent transcripts paired with their DNA template could influence transcription *via* binding to NF90 family proteins. The demonstration that the activity of NF110b in gene expression assays is controlled by its interactions with a structured RNA,[27] taken together with the recent discovery of several regulatory RNAs,[49–52] indicates that NF90's activity could be dictated by its ability to interact with various cellular regulatory RNAs. Interestingly, the ability of NF90 to bind VA $RNA_{II}$[24] suggests that the virus may be acting to inhibit the ability of this protein family to bind cellular RNAs, thereby possibly perturbing cell function to the advantage of the virus.

NF45, the heteromeric partner of NF90, acts as a co-activator to support NF90-mediated transcription.[3,22] Immunodepletion experiments with NF90 and NF45-specific antibodies indicated that both proteins are required for activated, but not basal, transcription from an ARRE-2 template *in vitro*.[3] The relative levels of NF90 and NF45 did not change when T-cells were activated, and NF45 is co-expressed with NF90 in several different cell types, indicating that the presence of both NF90 and NF45 is not sufficient to mediate activation of the IL-2 gene.[4] Similarly, in *Xenopus* where the CBTF homologues of NF90 were isolated as putative regulators of GATA-2 expression,[11,12] the CBTFs are expressed in several tissues that do not express the GATA-2 gene, suggesting that additional co-factors are required for function.[11] Most likely these putative additional co-factors dictate the specificity of NF90/NF110 function.

In summary, members of the NF90 family have distinct abilities to regulate gene expression. Their activity is modulated by association with NF45 and additional co-factors and enhancer elements, as well as by RNA regulators and, most likely, post-translational modifications. The contribution of each of the NF90 isoforms in regulating cell function has yet to be deciphered. It remains to be determined whether these proteins serve redundant functions in cells or if they carry out unique independent processes. A detailed assessment of the specificity of these different isoforms of NF90/NF110 and their possible relationship to different patterns of gene expression will help to further define their role in mammalian cell physiology.

## Materials and Methods

### Cloning of NF90 and NF110 isoforms and plasmid constructs

For gene-specific amplification of NF90 and NF110, PCR reactions were performed using a placenta cDNA library (Clontech) with the following primers: 5′-CCAGGGCTGCAGTACAAGCTGGTGTCCC (NF90-F), 5′-GCAATACTTTTAGACGCTCTAGGAAGAC (NF90-R) or 5′-GTGGCGAAGGCCTACGCTGCTCTTGCTGCC (NF110-F) and 5′-CGCGGGGGCTTATCTGTACTGG (NF110-R).

The resulting fragments were ligated into the pCRII-Topo cloning vector and sequenced to verify the presence and correctness of the inserts. The N terminus of NF90/NF110 was amplified using the placenta cDNA library plus the indicated primers: 5′-CAGAAGAAGTAAAATGCGTCCAATGCG and 5′-AGCAAGAGCAGC GTAGGCCTTCGCCAC.

The resulting product was ligated into the pCRII-Topo cloning vector to create pCRII-NF90n.

pcDNA3.1-NF90c was previously described as pcDNA3.1-NF90.[22] To create pcDNA3.1-NF90a, pCRII-NF90 and pCRII-NF90n were digested with *Bam*HI and *Bsa*AI or *Bsa*AI and *Eco*RI, respectively. Fragments were gel-purified and ligated into pcDNA3.1(+) digested with *Eco*RI and *Bam*HI. For pcDNA3.1-NF90b, pCRII-NF90 pCRII-NF90n was digested with *Hin*dIII and *Bam*HI or *Eco*RI and *Hin*dIII, respectively. The two fragments were gel-purified and ligated into pcDNA3.1(+) digested with *Eco*RI and *Bam*HI. To create pcDNA3.1-NF110b, pCRII-NF110 was digested with *Hin*dIII and *Bst*XI, and ligated with the NF90n (*Eco*RI/*Hin*dIII-digested) into pcDNA3.1(−) digested with *Eco*RI and *Bst*XI. To create pcDNA3.1-NF110a, pcDNA3.1-NF90 was digested with *Eco*RI and *Hin*dIII. The resulting fragment was gel-purified and ligated with NF110 (*Hin*dIII/*Bst*XI) into pcDNA3.1(−) digested with *Eco*RI and *Bst*XI. All of the resulting plasmids were verified by sequencing. To create the His/X-press (omni)-tagged expression plasmids, all inserts were digested with *Eco*RI and ligated into a similarly digested pcDNA3.1-HisB plasmid (Invitrogen). The resulting plasmids were screened for orientation.

To create pcDNA3.1-NF110bΔ336N, pcDNA3.1-NF110b was digested with *Eco*RV and *Eco*RI. The *Eco*RI site was then filled in with Klenow fragment and the vector was religated to remove the intervening sequence.

### Cell fractionation and Western blot analysis

Human lymphocytic Jurkat cells were grown in RPMI medium (Invitrogen) with 10% (v/v) fetal bovine serum (FBS, Sigma) and 1% (w/v) penicillin/streptomycin solution (P/S, Invitrogen) and fractionated as described.[53] Briefly, $2\times10^7$ cells were pelleted, washed twice with ice-cold phosphate-buffered saline (PBS), and divided into two (one for treatment with micrococcal nuclease). Each cell pellet was resuspended in 500 μl of buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% (v/v) glycerol, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 μg/ml of pepstatin A, 0.1 mM benzamidine) containing 0.1% (v/v) Triton X-100 for five minutes on ice. Following lysis, nuclei were pelleted at 1100*g* for five minutes and the supernatant removed (S2). S2 was further clarified by centrifugation at 17,500*g* for ten minutes. The remaining pellet (P2) was washed once with 1 ml of buffer A. Nuclei were resuspended in 500 μl of buffer A containing 1 mM $CaCl_2$ and 0.4 micromolar units of micrococcal nuclease (MNase) was added to one tube. Both samples were incubated at 37 °C for three minutes, and digestion was stopped by the addition of 2.5 μl of 0.2 M EGTA. Nuclei were pelleted at 1100*g* for five minutes, then lysed in 500 μl of buffer B (3 mM EDTA (pH 8.0), 0.2 mM EGTA (pH 8.0), 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 μg/ml of pepstatin A, 0.1 mM benzamidine) on ice for 30 minutes. Samples were then centrifuged at 6600*g* for five minutes, and the supernatant was removed (S3 ± MNase). S3 was further clarified by centrifugation at 17,500*g* for ten minutes. The resulting pellet was washed with 1 ml of buffer B, and then resuspended in 500 μl of buffer B. For total cell extract (TCE), $1\times10^7$ cells were pelleted, washed twice with ice-cold PBS, and lysed in 250 μl of buffer A with 62.5 μl of 4× Laemmli sample buffer (LB).

For Western blots, equal volumes (30 μl) of each cell fraction with LB were heated to 100 °C for five minutes and resolved by SDS-PAGE. Proteins were then transferred to nitrocellulose and probed with antibodies against NF90 and NF45,[4] and TRAF2 (Santa Cruz). Proteins were visualized by incubating blots with enhanced chemiluminescence (ECL) solution followed by exposure to film. To detect histones, 2 μl of each sample was separated in an SDS/15% polyacrylamide gel, and stained with Coomassie brilliant blue.

### Protein purification and mass spectrometry

Nuclei were isolated from a 13 l suspension culture of HeLa cells (NIH) grown in minimal essential medium (MEM; Mediatech Inc.) supplemented with 5% (v/v) calf serum and 2 mM L-glutamine, to a density of $5\times10^5$ cells/ml. Cells were harvested, washed in chilled PBS, resuspended in chilled hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 100 μg/ml of PMSF, 0.5 mM DTT), and disrupted with a Dounce homogenizer. Nuclei were pelleted at 3300*g* and extracted with high-salt buffer (20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.6 M KCl, 50 mM NaF, 1 mM $Na_3VO_4$, 100 μg/ml of PMSF, 0.5 mM DTT) for 30 minutes at 4 °C. Debris was pelleted at 25,000*g* and extracts were dialyzed for two hours at 4 °C against a solution containing 20 mM Hepes (pH 7.9), 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 100 μg/ml of PMSF, 0.5 mM DTT, then centrifuged at 10,000*g* and immediately frozen at −80 °C.

For protein purification, 2 ml (50% (v/v) suspension) of poly(I:C)-Sepharose resin (Pharmacia Biotech) was gravity-fed into a disposable column (0.7 cm diameter; BioRad) and washed five times with 1 ml of a solution containing 200 mM NaCl and 20 mM sodium phosphate (pH 7.5). HeLa nuclear extract was diluted into equilibration buffer (20 mM Hepes (pH 7.6), 3 mM $MgCl_2$, 200 mM KCl, 20% glycerol, 0.2 μg/ml of leupeptin, 0.2 μg/ml of aprotinin, 0.1 μg/ml of pepstatin A, 100 μg/ml of PMSF, 0.5 mM DTT) and loaded five times under low pressure. After a single wash (2 ml) with equilibration buffer containing 0.01% (v/v) Triton X-100, bound proteins were eluted with a linear gradient of 0–2 M NaCl in equilibration buffer containing 0.01% Triton X-100, using a simple gradient-maker coupled to a peristaltic pump. Eluate fractions (typically 20 × 0.4 ml) were collected by an automated fraction collector. Fractions containing NF90 and NF110 proteins were identified by Western blot analysis using a rabbit polyclonal antibody (Covance) raised against a peptide corresponding to the NF90 amino acid sequence EGRDSSKGEDSA-EETEAKPA (Research Genetics).

For NF90c, a cell line stably producing this protein was established by transfecting 293 cells with pcDNA3.1-NF90c plasmid expressing 5′- omni tagged NF90c. Monolayer cultures were grown to 80% confluency in high-glucose Dulbecco's MEM (DMEM; Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS; Sigma). Cells were lysed with a solution containing 50 mM Hepes (pH 7.9), 50 mM NaCl, 2 mM $MgCl_2$, 0.5% (v/v) NP-40, 50 mM NaF, 1 mM $Na_3VO_4$,

0.5 mM EDTA, 0.2 mM EGTA, 10% glycerol, 0.5% (w/v) SDS, 0.2 μg/ml of leupeptin, 0.2 μg/ml of aprotinin, 0.1 μg/ml of pepstatin A, 100 μg/ml of PMSF, 0.5 mM DTT. The lysate was frozen on solid $CO_2$, diluted fivefold with respect to SDS, and the chromosomal DNA sheared mechanically. After clarification of the lysate by centrifugation at 10,000*g* for ten minutes, ectopically expressed NF90c was extracted from the supernatant by immunoprecipitation with omni-probe antibody (Santa Cruz Biotech.).

Protein samples were boiled in LB and resolved in a 1 mm, SDS/7.5% polyacrylamide gel. Protein bands were visualized by staining with Simply Blue Safe Stain (Invitrogen), excised, diced into 1 $mm^3$ pieces, and transferred to pre-washed tubes (60% (v/v) $CH_3CN$, 0.1% (v/v) trifluoroacetic acid). Gel pieces were washed in 50% $CH_3CN$ for 30 minutes, then incubated in 10 mM DTT, 100 mM $NH_4HCO_3$ for 45 minutes at 56 °C to reduce the protein. Cysteine residues were alkylated by incubation for 30 minutes at room temperature in the dark in 55 mM iodoacetamide, 100 mM $NH_4CO_3$. To remove stain, gel pieces were washed in 50% $CH_3CN$, 50% 50 mM $NH_4HCO_3$ for 30 minutes, then in 50% $CH_3CN$, 50% 25 mM $NH_4HCO_3$ for 30 minutes, and were dried *in vacuo*. For digestion, 25 mM $NH_4HCO_3$ solution containing 10 ng/μl of modified trypsin (Promega) was added. After incubation overnight at 37 °C, tryptic peptides were extracted from the gel pieces by a combination of repeated vortex mixing and sonication in 60% $CH_3CN$, 5% trifluoroacetic acid. Peptides were desalted and partially purified on a $C_{18}$ ZipTip (Millipore), before elution onto α-cyano-4-hydroxycinnamic acid (Sigma) matrix in readiness for matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy analysis. All mass spectra were acquired in positive-ion reflector mode. The on-line programs Protein Prospector (University of California, San Francisco) and Profound (Rockefeller University, NY) were used in sequence database searches of the NCBI and Swiss-Prot data banks.

### Cell transfection and reporter assays

HeLa cell monolayers were grown in DMEM (Sigma) and 10% FBS and 1% P/S. Cells were seeded in 12-well plates at $1.5 \times 10^5$–$1.75 \times 10^5$ cells/well at ~12 hours prior to transfection. For transfection, the medium from each well was replaced with a transfection cocktail prepared as follows. The indicated plasmids were diluted in 50 μl of serum-free DMEM with 2.5 μl of Cytofectene (Biorad), incubated at room temperature for 20 minutes, then diluted with 450 μl of medium. Cells were harvested at 48 hours after transfection as indicated for the different reporter assays.

For CAT assays, cells were washed twice with ice-cold PBS, and lysed by freeze-thawing (three times) in 150 μl of 0.25 M Tris–HCl (pH 7.6). Extracts were clarified by centrifugation at 17,500*g* for ten minutes. Approximately 5–75 μl of extract was assayed per sample in each reaction, depending on the basal activity of the promoter (usually 5 μl for the −1265 PCNA promoter and 75 μl for the −46 PCNA promoter). Reactions were carried out for 30 minutes as described.[29] Samples with <1 or >40% conversion were repeated with more or less extract to ensure that all the reactions were in the linear range. CAT acetylation values were normalized to total protein.

To harvest cells for firefly luciferase or β-galactosidase assays, cells were washed twice with ice-cold PBS and lysed in 150 μl of reporter lysis buffer. Reporter assays were carried out with 20 μl of lysate as described.[22]

### RNase protection assay

The β-galactosidase probe was synthesized using the pBI-gal plasmid, which contains the full-length β-galactosidase gene in anti-sense orientation downstream of a phage T7 promoter. This plasmid was digested with *Eco*RV and used as a template in an *in vitro* transcription reaction using phage T7 RNA polymerase as described.[22] The probe was gel-purified and eluted for subsequent reactions. HeLa cells were seeded at $3 \times 10^7$ cells/well in six-well plates and grown as described above. Cells were transfected using Cytofectene with 400 ng of SV40-βgal reporter plasmid plus 1.6 μg of NF110b vector or pcDNA3.1 empty vector. Cells were harvested after 24 hours. RNA isolation and RNase protection assays were carried out as described.[22] Results were obtained by autoradiography and quantified using an Alphaimager (Alphainnotech).

### NF110b/RHA coimmunoprecipitation

For analysis of native complexes, 293 cell extracts were prepared by homogenization.[24] Nuclear fractions (500 μg) were treated with RNase A at 300 μg/ml for one hour at 30 °C, then incubated at 4 °C in the presence of 5–10 μl of antibody for two hours with rocking. Immune complexes were collected using protein A-Sepharose beads (Pharmacia) and washed five times with 500 μl of buffer C (20 mM Hepes–KOH (pH 7.4), 200 mM KCl, 0.1% NP40, 0.5 mM DTT). The beads were resuspended in 30 μl of 2 × LB, boiled for three minutes, resolved by SDS-PAGE, and subjected to Western blot analysis using anti-NF90[4] and anti-RHA[38] antibodies both at 1:3000.

For complexes containing wild-type and mutant NF110b proteins, HeLa cells (~70% confluent) were transfected with 500 ng of the indicated expression plasmid plus 1.1 μg of empty pcDNA3.1 in a six-well plate as described above. After 36 hours, cells were lysed in 300 μl of RIPA lysis buffer (10 mM Tris–HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM NaF, 0.5 mM PMSF, 0.1 mM DTT, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 μg/ml of pepstatin A, 0.1 mM benzamidine) for 30 minutes at 4 °C. Lysates were clarified by centrifugation at 17,500*g* for ten minutes. For immunoprecipitation, 2.5 μl of omni-probe antibody was added to each tube and incubated with agitation for two hours at 4 °C. Then 5 μl of packed protein A-Sepharose was added to each tube and incubated for an additional hour. Immunoprecipitates were washed four times with RIPA buffer, resuspended in 5 μl of 4 × LB, and separated in an SDS/7% polyacrylamide gel. Proteins were transferred to a 0.45 μm pore size nitrocellulose membrane, probed with anti-RHA antibody (1:3000), and visualized by ECL and autoradiography. For anti-omni Western blots, membranes were stripped using the Re-blot Western Blot Recycling kit (Chemicon) and reprobed using the omni probe (1:1000, Santa Cruz Biotech).

### Poly(I:C) pulldown assay

Proteins were synthesized using the TnT T7 coupled rabbit reticulocyte lysate (Promega) in the presence of

[35S]Translabel (ICN Pharmaceuticals). For binding assays, 4 μl of each TnT product was diluted in 50 μl of binding buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 1 mM DTT, 0.5 PMSF), then added to 10 μl of packed poly(I:C)-Sepharose beads (Amersham Pharmacia Biotech). Samples were incubated for 40 minutes at room temperature with rocking, and washed five times with binding buffer, or with binding buffer supplemented to contain a concentration of 300 mM, 500 mM or 800 mM NaCl. A final wash was performed with binding buffer. The beads were then resuspended in LB, boiled, and resolved in an SDS/8% polyacrylamide gel. Gels were dried and quantified using a Packard Instant Imager.

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