Constitutive silencing of IFN-β promoter is mediated by NRF (NF-κB-repressing factor), a nuclear inhibitor of NF-κB

Mahtab Nourbakhsh and Hansjörg Hauser[1]

Department of Gene Regulation and Differentiation, GBF–National Research Institute for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

[1]Corresponding author
e-mail: hha@gbf.de

Transcriptional regulation of the interferon-β (IFN-β) gene is characterized by strict constitutive repression and virus-specific activation. Previous studies have shown that the IFN-β promoter is constitutively repressed by a negative regulatory element (NRE). Isolated NRE acts as a constitutive and position-independent silencer on the NF-κB-binding sites. Here, we describe the identification and functional characterization of the NRE-binding protein, called NRF (NF-κB-repressing factor), which abolishes the transcriptional activity of the bordering NF-κB- binding sites by a distance-independent mechanism. Deletion studies show that a minimal repression domain of NRF is sufficient to exert its inhibitory effect. *In vitro*, NF-κB proteins bind to purified NRF by a direct protein–protein interaction. We demonstrate that NRF is a ubiquitous and constitutive nuclear protein. In fibroblasts, the expression of the NRF antisense RNA releases the endogenous IFN-β gene transcription. Our data strongly suggest that the NRF-mediated inhibition of NF-κB is a critical component of the IFN-β gene silencing prior to viral infection.
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Introduction

The highly specific activation of the interferon-β (IFN-β) promoter in response to virus infection requires an overlapping set of the regulatory elements termed positive regulatory domains (PRDI–PRDIV). PRDII, PRDI–III and PRDIV are recognized by the transcription factors NF-κB, IRF-1 and ATF-2/c-Jun heterodimers, respectively (Thanos and Maniatis, 1995a). Maximal activation of the IFN-β promoter requires the cooperative binding of these transcription factors together with the high mobility group protein HMGI(Y) which form a multicomponent complex, termed the enhanceosome (Thanos and Maniatis, 1995b; Kim and Maniatis, 1998). Recent studies have shown that the transcriptional synergy within this complex requires the recruitment of the CBP/p300 coactivator (Merika et al., 1998). The final transcriptional complex is formed in the presence of TFIID, A and B and cofactor USA.

In virus-induced cells, three negative regulatory proteins have been identified modulating the activity of PRDs in the IFN-β promoter. IRF-2 and PRDI-BF1/Blimp-1 were described to bind to PRDI (Harada *et al*., 1989; Keller and Maniatis, 1991), while PRDII-BF1 was isolated by binding to PRDII (Fan and Maniatis, 1989). The virus-inducible expression kinetics of these proteins have established that they are involved in the post-induction shut off mechanism of the IFN-β promoter. For example, in IRF-2-deficient mice, the virus-induced level of IFN-β mRNA was shown to be higher than the level observed in wild-type mice (Matsuyama *et al*., 1993). Similarly, a dominant-negative PRDI-BF1 significantly increases the virus-inducible transcription of the IFN-β gene (Ren *et al*., 1999).

Apart from the post-induction shut off mechanism, the human IFN-β promoter was shown to be constitutively repressed by a negative regulatory domain (NRD). The NRD was defined by the successive deletion of the 5′ regulatory region of the IFN-β gene which led to activation of the IFN-β promoter (Goodbourn and Maniatis, 1988). Within the NRD, we have identified a negative regulatory element (AATTCCTCTGA), called the NRE (Nourbakhsh *et al*., 1993). Deletion or distinct point mutations of the NRE sequence also led to the virus-independent stimulation of the IFN-β promoter. Detailed studies of the NRE-mediated silencing revealed that this element specifically interacts with PRDII, the NF-κB-binding site in the IFN-β promoter. NF-κB, primarily identified as a transcription factor that regulates the κ-light chain expression in B-lymphocytes, exists in most cell types as homodimers or heterodimers of a family of structurally related proteins (May and Ghosh, 1997). To date, five NF-κB proteins have been identified: p65 (RelA), c-Rel, RelB, p50 (NF-κB1) and p52 (NF-κB1). A large and growing number of extracellular signals induce a signaling cascade that increases the nuclear concentration of the NF-κB dimers transiently. Nonetheless, NF-κB proteins were found to be constitutively present in the nuclei of different cell types and to bind to their respective sites (Miyamoto *et al*., 1988; Grilli *et al*., 1993; Kopp and Ghosh, 1994). In fact, the isolated NF-κB-binding sites, like the PRDII of the IFN-β promoter, act as constitutively active transcriptional enhancers (Fan and Maniatis, 1989; Nourbakhsh *et al*., 1993). However, the intact IFN-α promoter, although bearing a functional NF-κB-binding site, is constitutively silent and displays a highly specific induction by virus but not by the other NF-κB stimuli. Thus, we assumed that an NF-κB-specific repression mechanism may contribute to the constitutive silencing of the IFN-β promoter and provide the high specificity of the virus induction.

Considering the characteristics of the NRE, we proposed that a constitutive transcriptional silencer binds to this element and represses the basal activity of the IFN-β promoter. Electrophoretic mobility shift assay (EMSA) and cross-competition experiments have established that the NRE forms a specific complex in the nuclear extracts

of non-induced cells (Nourbakhsh *et al*., 1993). Contrary to our expectations, the NRE-specific complex was also observed in virus-stimulated cells. Consistent with this, we found that in virus-induced cells the NRE is still capable of silencing the transcriptional activity of the isolated PRDII. Based on these observations, we suggested that the concentration, binding affinity and PRDII silencing capacity of the NRE-binding silencer protein are not altered during viral infection. This apparent discrepancy was finally resolved by the experiments showing that in the intact IFN-β promoter, the viral induction results in a synergistic interaction between PRDI and PRDII which is not affected by the simultaneous binding of the NRE-binding factor (Nourbakhsh *et al*., 1993). Indeed, EMSA experiments revealed the simultaneous binding of the nuclear factors to the PRDs and NRE. Furthermore, these results suggested an active but not a competitive repression mechanism by the NRE-binding factor. However, direct proof has not been obtained because the binding factor was not identified.

Here, we describe the cloning, characterization and functional tests of an NRE-binding protein. This protein, termed 'NF-κB-repressing factor' (NRF), specifically inhibits the transcriptional activity of the NF-κB proteins, probably by a direct protein–protein interaction. Most importantly, NRF by its apparent inhibitory effect on NF-κB is engaged in the constitutive silencing of the IFN-β promoter.

Results

Cloning of the NRE-binding factor

NRE is an 11 bp sequence element which was shown to be sufficient for the silencing of the IFN-β promoter and binding of nuclear factors in EMSA and UV cross-linking experiments (Nourbakhsh *et al*., 1993). To determine the number and size of nuclear proteins which bind to the NRE, we carried out South-Western blot analysis. Using monomeric NRE, two distinct bands were observed in nuclear extracts from HeLa cells (Figure 1A, lane 2) corresponding to mol. wts of ~100 and 50 kDa. Under the same conditions, bacterial proteins isolated from the screening strain failed to bind to the NRE (Figure 1A, lane 1). Screening of 2.5×10^6 recombinants of a HeLa cDNA expression library revealed 36 different NRE-binding clones. However, in control experiments, only one of the 36 identified clones (Figure 1B) failed to bind to a functional inactive mutant of NRE (Nourbakhsh *et al*., 1993). Sequence analysis of the cDNA insert revealed that it encodes the C-terminal 92 amino acids of an open reading frame (Figure 1D), indicating that the encoded region constitutes the DNA-binding domain of the NRE-binding protein. Interestingly, the predicted secondary structure of this region revealed four turns and four α-helices which are arranged similarly to those in the wHTH family (winged helix–turn–helix) of DNA-binding motifs (Donaldson *et al*., 1996).

Using the C-terminal cDNA as a probe, additional hybridization screening was carried out to obtain the complete cDNA sequence. The predicted molecular weight of the protein, called NRF, is 43.8 kDa. However, the *in vitro* translated NRF shows an apparent size of ~50 kDa, which corresponds to the fast migrating protein
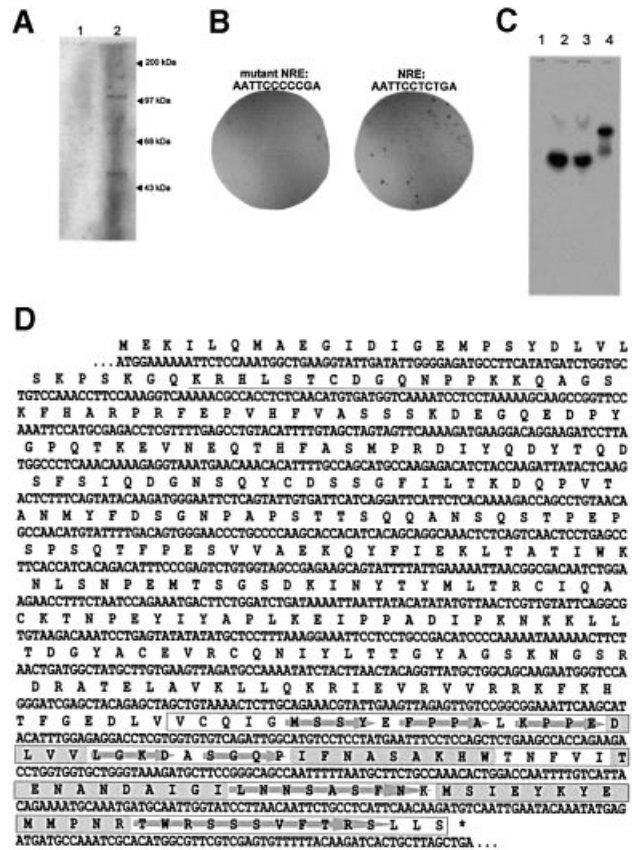

Fig. 1. Identification and cDNA cloning of NRF. (**A**) Nuclear proteins which bind to the IFN-β NRE. A 10 μg aliquot of *Escherichia coli* Y1090 (lane 1) and 10 μg of HeLa cell nuclear extract (lane 2) were separated by gel electrophoresis. Proteins were transferred to an NC filter, incubated with ^{32}P-labeled NRE probe. The size of the detected bands was calculated from the co-electrophoresed marker proteins indicated (right). (**B**) Filter-binding assay of the λ phage encoding the NRF DBD. A total of 10^8 permissive cells of the Y1090 strain were infected with 100 recombinant λZapII phages containing the NRF cDNA. Infected cells were overlaid with an IPTG-impregnated NC filter. After incubation for 3 h, the filter was removed and replaced by a second filter and incubated again for 3 h. The first filter was incubated with the labeled mutant NRE probe (left) and the second filter with the wild-type NRE probe (right). (**C**) Identification of the NRF-containing complex in HeLa cell nuclear extract. A 2.5 μg aliquot of nuclear extract from HeLa cells was incubated with ^{32}P-labeled NRE probe for 10 min at room temperature. Pre-formed complexes were incubated for 30 min with 10 μg of rabbit pre-immune serum (lane 3), with 10 μg of antiserum mixture directed against NRF peptide sequences (lane 4) and with buffer alone (lane 2). The unbound probe incubated with antiserum against NRF was run off the gel to facilitate resolution of the complexes (lane 1). (**D**) Nucleotide sequence of the NRF reading frame and the deduced protein sequence. The nuclear localization sequence is underlined and the DNA-binding domain is indicated by the dotted box. The indicated turn (arrows) and α-helix regions (shaded boxes) were calculated by the Robson prediction method. The complete cDNA sequence and the derived amino acid sequence of NRF have been deposited in the DDBJ/EMBL/GenBank database (accession No. AJO11812).

in the South-Western experiment in Figure 1A (data not shown).

To determine whether NRF is a component of the NRE-specific complex in nuclear extracts, supershift experiments were performed using a mixture of rabbit polyclonal antipeptide antibodies directed against NRF. As shown in Figure 1C, NRE forms a specific complex in the nuclear extract from HeLa cells (lane 2). The anti-
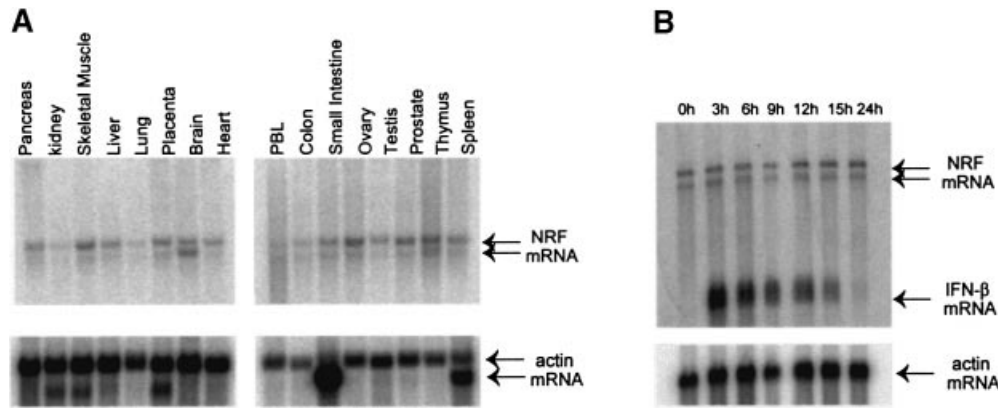
Fig. 2. Expression pattern of NRF mRNAs. (**A**) Expression of NRF mRNA in various human tissues. Upper panel: the RNA blot was hybridized with full-length NRF cDNA. Lower panel: the blot was washed and hybridized with a β-actin probe. The origin of the tissue in each lane is indicated on the top. (**B**) Expression of NRF mRNAs in virus-induced cells. HeLa cells were induced with Sendai virus for 1 h and poly(A) RNA was extracted at the indicated times after induction began and subjected to Northern blot analysis using full-length cDNA encoding NRF and IFN-β as probes. Each lane received 2 μg of poly(A) RNA. NRF, IFN-β and β-actin signals are indicated by arrows.

NRF antisera recognize this complex efficiently (lane 4), but not the unbound NRE probe (lane 1). In contrast, addition of pre-immune sera has no effect on the migration of the NRE-specific complex (lane 3). These results demonstrate that NRF is a predominant component of the nuclear proteins binding to NRE.

Expression of NRF mRNAs

The IFN-β transcription was shown to be ubiquitously repressed (Goodbourn and Maniatis, 1988). To address whether the expression pattern of NRF correlates with the ubiquitous repression of the IFN-β gene, we have monitored NRF mRNA in a variety of human tissues. As shown in Figure 2A, two 3.7 and 4.0 kb mRNAs were detected in all tested tissues. Accordingly, by the screening of the HeLa cDNA library, we have identified two NRF-encoding cDNAs which differ in the length of their 3′-untranslated regions (UTRs). The shorter 3′ UTRs lacked 940 bp. Gene mapping analysis revealed that NRF is encoded by a single-copy gene, suggesting that the generation of two mRNAs is due to differential poly-adenylation (data not shown).

The results presented in Figure 2A show that the NRF mRNAs are detected in all tested tissues. However, the strength of NRF mRNA expression varies slightly amongst the different tissues. NRF is expressed to a lower extent in colon, peripheral blood lymphocytes, lung and kidney, and the small 3′ UTR variant is more abundant in brain and thymus and low in heart, pancreas and spleen.

Previous EMSA experiments have suggested that the expression of the NRE-binding factor is not altered by viral infection during the induction of the IFN-β gene (Nourbakhsh *et al.*, 1993). To confirm this, we examined the expression level of NRF mRNAs in response to viral induction. As shown in Figure 2B, IFN-β mRNA is induced 3 h after viral infection and disappears within the following 24 h. In contrast, the amount of both NRF mRNAs is not altered during this time. Taken together, these results confirm the previous assumptions and demonstrate the constitutive and ubiquitous expression of the NRF.

Nuclear localization of NRF

To investigate the nuclear localization of NRF, we have used an N-terminal fusion of green fluorescent protein
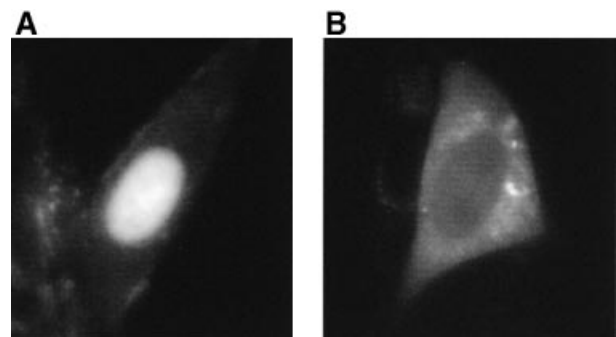
Fig. 3. Nuclear localization of NRF. (**A**) Transient C243 transfectants expressing the GFP–NRF fusion protein. (**B**) Transient C243 transfectants expressing fusion protein composed of the GFP and the deletion mutant of NRF lacking the nuclear localization signal amino acids 25–45. Cells were subjected to fluorescence microscopy and photographed.

(GFP) (Misteli and Spector, 1997; Schaper *et al.*, 1998). Figure 3A shows that the GFP–NRF fusion protein is constitutively localized in the nucleus. In all GFP–NRF-expressing cells, this pattern was observed evenly following viral induction (data not shown). Within the N-terminal domain of NRF, a short sequence of 16 amino acids is closely related to the nuclear localization signal consensus (Dingwall and Laskey, 1991). As shown in Figure 3B, the deletion of this sequence results in the predominant localization of the mutant protein in the cytoplasm. These data show that NRF contains a functional nuclear localization signal which is responsible for its permanent recruitment into the nucleus.

DNA-binding activity of NRF

To examine the DNA-binding ability of NRF *in vivo*, we have carried out reporter gene experiments. Reporter plasmids p0, pNm and pN contain a CAT expression cassette driven by a minimal TATA-box promoter. pN and pNm harbor an NRE or a mutant NRE, respectively, positioned 5′ to the TATA-box. p0, pNm and pN were transfected simultaneously with effector plasmids coding for NRF–VP16 fusion proteins. pDBDVP16 encodes a fusion protein of the NRF DNA-binding domain (DBD) and the VP16 transactivation domain. The VP16 transactivation domain (pVP16) alone and NRF (pNRF)

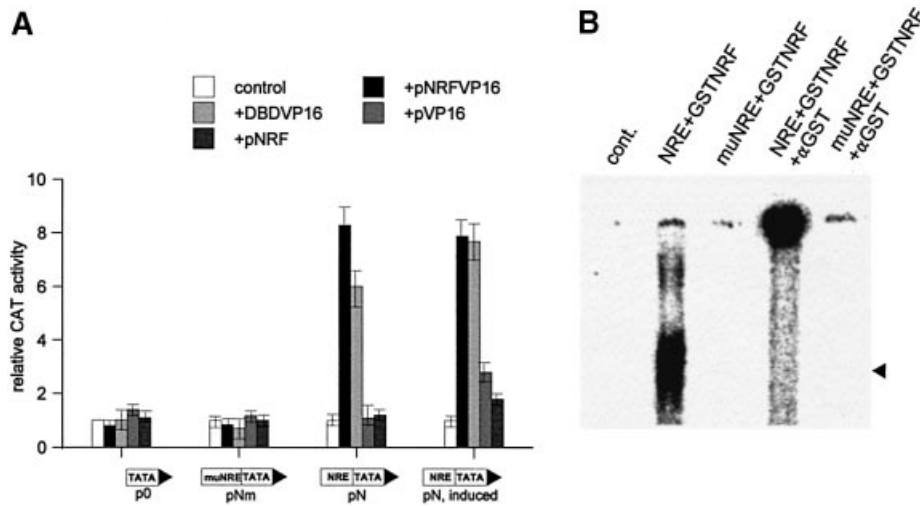**Fig. 4.** DNA-binding activity of NRF. (**A**) C243 cells were transfected with the indicated expression and reporter plasmids together with a luciferase expression plasmid as an internal standard. CAT and luciferase expression were determined and the relative CAT activity was calculated. The data were obtained in at least six independent transfection experiments. The p0 reporter gene (CAT) activity was adjusted to 1. The p0 reporter is driven by a truncated HSV-TK promoter, pN contains an additional NRE sequence and pNm contains the mutant NRE sequence upstream of the TK promoter. 'pN induced' indicates that the cells were induced by Sendai virus 24 h after transfection and incubated for a further 24 h. Black and dotted bars show transfection assays with additional effector plasmids. Control indicates empty vector; pDBDVP16 encodes a fusion protein composed of the NRF DBD (amino acids 296–388) and the VP16 transactivation domain; pNRF encodes wild-type NRF; pNRFVP16 encodes a fusion protein composed of NRF and the VP16 transactivation domain; pVP16 encodes the VP16 transactivation domain. (**B**) Gel shift assay with ^{32}P-labeled double-stranded NRE or mutant NRE oligonucleotide and extracts from IPTG-induced recombinant *Escherichia coli* strain BL21 expressing the GST–NRF fusion protein. The components in each lane are indicated at the top. In the left two lanes, the pre-formed mixtures were incubated with 1 μg of GST-specific antibody. In the control lane (cont.), no nuclear extract was added to the mixture. The closed triangle indicates the NRF-specific complex without the addition of antibodies. The unbound probe was run off the gel to facilitate resolution of the complexes.

were used as negative controls. As shown in Figure 4A, NRF–VP16 and DBD–VP16 stimulate the expression of the reporter gene pN, whereas NRF or Vp16 expression did not alter the activity of the same reporter. Furthermore, the expression level of reporter plasmids p0 and pNm which do not contain a functional NRE sequence are not enhanced by VP16 fusion proteins. To examine the NRE-binding capacity of NRF–VP16 in response to the viral induction, cells were induced following the transfection. The data show that the enhancing effect of NRF–VP16 or DBD–Vp16, and thus the binding activity of NRF, is not altered by viral infection.

The experiments described above do not exclude the need for additional nuclear proteins for binding of NRF to the NRE. To address this possibility, gel shift experiments were performed using the purified GST–NRF fusion protein (Figure 4B). The purified GST–NRF fusion protein is able to bind to the NRE probe but not to the mutant NRE probe. The GST–NRF-specific complex is detected using antibodies directed against the GST domain. The results demonstrate that additional nuclear factors are not required for the binding of NRF to the NRE sequence. Importantly, the DNA-binding specificity of the cloned NRF correlates *in vivo* and *in vitro* with the binding specificity of the endogenous NRF protein which was characterized previously (Nourbakhsh *et al.*, 1993).

Derepression of the endogenous IFN-β gene

As direct proof of the role of NRF in the constitutive repression of the IFN-β gene, we have measured the production of endogenous interferon in response to NRF antisense expression (Figure 5A). The Tet-off expression system (Gossen and Boujard, 1992) was applied to exclude unspecific interferon induction which might be caused by

transient transfection (Pine *et al.*, 1988). We have therefore created pools of stable transfectants in which sense or antisense sequences of NRF RNA are expressed under the control of a tetracycline-responsive promoter. The absence of tetracycline leads to a low but significant expression of endogenous IFN-β in cells (Figure 5A), due to the expression of antisense NRF and the reduction of NRF expression (Figure 5D). The expressed antisense RNA is complementary to the human and murine NRF 5′-coding sequence (data not shown). In contrast, neither the expression of the full-length NRF nor the control plasmid without insert interfere with the endogenous IFN-β expression. The results were confirmed in the presence of 2-aminopurine, a potent inhibitor of the dsRNA- or poly(I):poly(C)-activated pathway (Ozes and Taylor, 1993). This excludes an unspecific effect of dsRNA which could result from the association of antisense RNA with endogenous mRNA.

To investigate whether the NRF expression level has an effect on the virus-induced expression of the endogenous IFN-β, the stable transfectants were stimulated by virus in the absence of tetracycline (Figure 5B). The data show that the level of NRF has no effect on the virus-induced expression of the endogenous IFN-β. This result is in agreement with previous reporter experiments demonstrating that the deletion of NRE has no effect on the virus-induced level of the IFN-β promoter (Nourbakhsh *et al.*, 1993).

To confirm that IFN-β expression is enhanced at the transcriptional level, we carried out Northern blot analysis. As shown in Figure 5C, IFN-β mRNA is detected specifically in cells which express the NRF antisense RNA. Together, these results strongly suggest that NRF is involved in the constitutive repression of the IFN-β promoter.
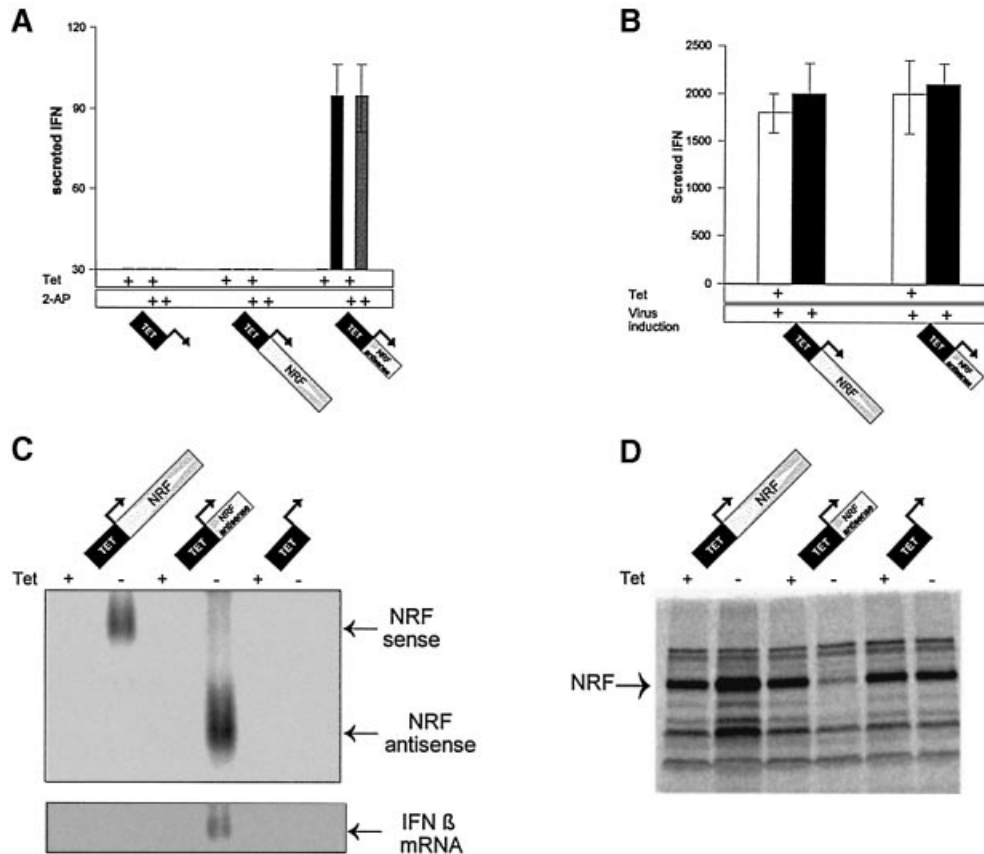
Fig. 5. Derepression of the IFN-β gene by NRF antisense RNA. (**A**) C243-TA cells were stably transfected with tetracycline-repressible expression plasmids encoding either full-length sense NRF RNA, 300 bp antisense NRF RNA or without insert. Stable transfectants were pooled and tested for interferon production in the presence (repressed) or absence of 2 µg/ml tetracycline (activated) for a period of 48 h. The data were obtained in at least three independent experiments. (**B**) For viral induction, cells were induced 24 h before testing the interferon production. Interferon activity is given as units per ml and 10^6 cells. AP indicates that 10 mM 2-aminopurine was present throughout the activation phase. (**C**) IFN-β mRNA expression. Poly(A)$^+$ RNA from cells treated as described above was analyzed by Northern blot analysis as described in the legend of Figure 2. (**D**) Pools of stable transfectants were tested for NRF expression. A 50 µg aliquot of the nuclear extracts was subjected to immunoprecipitation using rabbit polyclonal antibodies directed against amino acids 256–272 and 272–288. The cleared extracts were analyzed by Western blot analysis using polyclonal antibodies directed against amino acids 25–45, 175–191 and 364–382 (see Materials and methods).

Silencing of the NF-κB-binding site by NRF

Previous studies have established that the NRE acts on NF-κB-binding sites, especially on PRDII of the IFN-β promoter (Nourbakhsh *et al.*, 1993). To investigate whether NRF mediates this inhibitory effect, we used a GAL4 reporter assay (Figure 6A). All reporter experiments presented in this study were performed in two murine cell lines, C243 and LMTK. Since the same conclusion was drawn using both cell lines, only the results obtained in C243 cells are presented here (Figure 6A). The NF-κB-binding sites of the IFN-β promoter in pP2 and pP2G2 confer a constitutive expression level of the reporter gene, as reported earlier (Nourbakhsh *et al.*, 1993). Co-expression of pGAL4NRF encoding the GAL4–NRF fusion protein (the GAL4 DBD fused to the N-terminal end of the full-length NRF) decreases the transcriptional activity of the pP2G2 reporter to the level of p0 expression. pP2 is not repressed by co-expression of NRF or GAL4–NRF, nor is pP2G2 repressed by co-expression of NRF. To test the specificity of NRF action for NF-κB-binding sites, two reporter plasmids were used containing IRF-1- and Sp1-binding sites (pP1G2 and pSP1G2, respectively). Both reporters are expressed to a higher level than p0,

indicating that endogenous activator proteins bind to IRF-1- and SP1-binding sites. The transcriptional activity of both reporters is not altered by simultaneous expression of wild-type NRF or by GAL4–NRF.

In contrast to the overlapping formation in the IFN-β promoter, the NF-κB- and NRF-binding sites in the pP2G2 reporter are separated, suggesting that NRF does not act by a competitive mechanism. This conclusion was confirmed by reporter experiments using the LexA–NRF fusion protein and pL6P2 reporter (Figure 6B). In pL6P2, LexA-binding sites are integrated at a distance of ~50 bp 3' to the NF-κB-binding site. Similarly to the GAL4 reporter experiments, LexA–NRF inhibits the 3'-located NF-κB-binding sites, emphasizing the distance- and position-independent mode of the NRF function.

To confirm that the constitutive transcriptional activity of PRDII is mediated by the nuclear content of NF-κB proteins in unstimulated cells, we performed supershift experiments using labeled PRDII and NF-κB-specific antibodies. As shown in Figure 6C, two major complexes were detected in the nuclear extract of unstimulated C243 cells (lane 5). The faster migrating complex is not affected by addition of NF-κB-directed antibodies or by

the competition with other NF-κB-binding elements (data not shown). Indeed, Thanos and Maniatis (1992) have shown previously that this fast migrating PRDII complex contains the HMGI(Y) protein. However, the constitutive activity of PRDII cannot result from HMGI(Y) binding, since this protein exerts no activating capacity on basal transcription (Yie *et al.*, 1999). The upper complex is clearly retarded by addition of αp50 and αp65, forming distinct supershifted complexes. The αp52 and αc-Rel antibodies fail to form an apparent retarded complex.

Compared with the control (Figure 6C, lane 5), the intensity of the NF-κB complex is reduced by αp52 and αp65, assuming that a very low amount of the respective proteins may be contained in the complex. Together, these results indicate that in unstimulated C243 cells, a low amount of NF-κB is present in the nucleus and binds to the PRDII element.

The additional experiments suggest that NRF contains an active repression domain which can be separated from the DNA-binding domain moiety of the protein. A
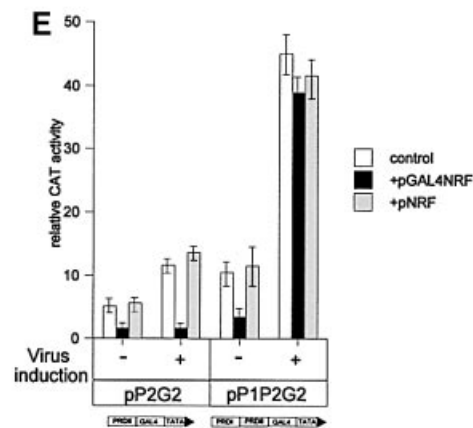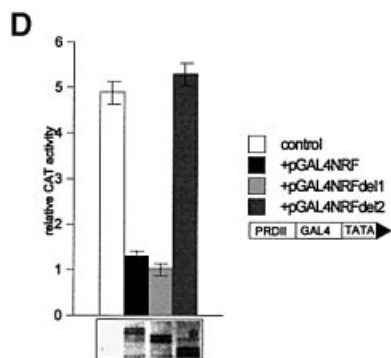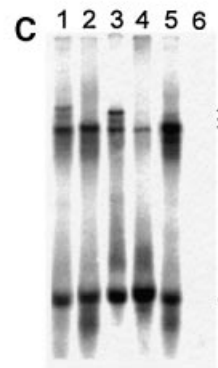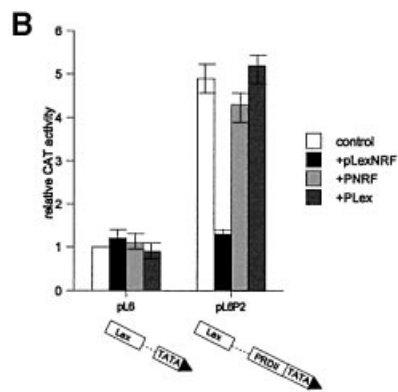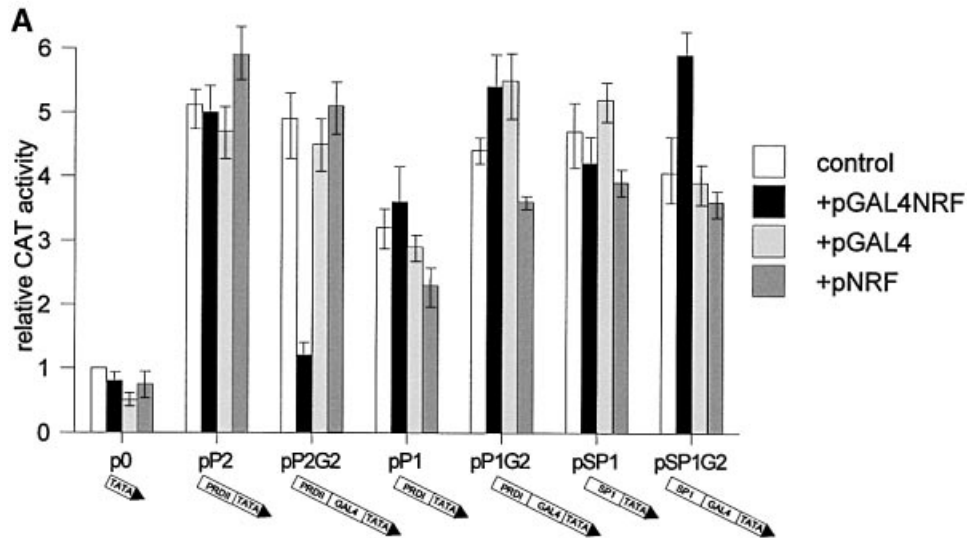
mutant fusion protein lacking the DNA-binding domain (amino acids 296–388) of NRF (GAL4–NRFdel1) behaves similarly to the GAL4–NRF (Figure 6D). Conversely, the GAL4–NRFdel2 protein containing only amino acids 1–193 of NRF has no effect on pP2G2 reporter activity. The expression levels of the GAL4–NRF fusion proteins are analyzed by Western blot analysis (Figure 6D, lower box). These results show that the C-terminal end of NRF (amino acids 1–296) acts as an active repression domain which can be fused to a foreign DNA-binding domain. Within the repression domain, a minimal region (amino acids 193–296) is essential for its function.

The transcriptional activity of NRF was also investigated in the virus-induced state. As summarized in Figure 6E, the viral induction results in a low stimulation of the NF-κB-binding sites (compare pP2G2 in non- or virus-induced cells). However, simultaneous expression of GAL4–NRF inhibits the constitutive or the virus-induced activity of the NF-κB-binding sites. This indicates that the silencing capacity of NRF is not affected by virus-induced signals. In pP1P2G2, the PRDI element of the IFN-β promoter is located 5′ to the PRDII element. This reporter is constitutively expressed. Similarly to pP2G2, the expression of GAL4–NRF decreases the transcriptional activity of pP1P2G2. However, in sharp contrast to pP2G2, the virus-induced activation of PRDII in concert with PRDI is not affected by GAL4–NRF. These data show that the synergistic interaction of PRDI and the NF-κB site is able to resist the inhibitory action of NRF. In agreement with the previous results, the synergistic interaction of PRDI and the NF-κB-binding site is only observed in virus-induced cells.

Interaction of NRF with NF-κB factors
The experiments described above taken together with previous reports strongly suggest that NRF interacts directly with the members of the NF-κB family. To address this point, we carried out GST pull-down experiments using purified GST–NRF fusion protein and ^{35}S-labeled NF-κB proteins, or IRF-1 and firefly luciferase as control (Figure 7A). All tested NF-κB proteins are able to bind to GST–NRF, but with distinguishable affinities. Whereas 10% of p50 or c-Rel proteins are recovered, only 5% of p52 and 2.5% of p65 proteins are co-purified by binding to

GST–NRF. IRF-1 and luciferase fail to bind to immobilized GST–NRF, showing the specificity of this binding assay. Furthermore, unspecific binding of labeled proteins to Sepharose was ruled out using parallel experiments without GST–NRF protein (control lanes).

Earlier studies have shown that p50/p65 heterodimers preferentially bind to the NF-κB site of IFN-β (PRDII) (Thanos and Maniatis, 1995a). However, other homo- and heterodimers also exhibit considerable affinity for PRDII. We have addressed the question of whether NRF shows a preference in the inhibition of distinct homo- or heterodimers of NF-κB (Figure 7B). The conclusions from these experiments can be summarized as follows. Co-expression of constructs encoding p65/p65, p65/c-Rel, p50/p65, c-Rel/c-Rel or p50/c-Rel activate the expression of the PRDII-containing promoter strongly above that elicited by the endogenous activators. In comparison, p50/p52, p52/p52 and p50/p50 cause a lower activation. Simultaneous expression of pGAL4NRF inhibits the level of transactivation mediated by all tested NF-κB dimers, but to a different extent. p65/p65, which is the most potent activator, is slightly inhibited. p50/p52 and p52/p52 are also slightly repressed but their activation capacity is negligible. The highest repression ratio was measured against p65/c-Rel-exerted activation. p50/p65, p52/c-Rel, p50/c-Rel, p52/p65, p50/p50 and c-Rel/c-Rel are also inhibited significantly. These data demonstrate first, the inhibitory effect of NRF on NF-κB dimers and second, the differential activation capacity of NF-κB factors in concert with NRF.

Discussion

The major pathway of NF-κB regulation exists in the cytoplasm, where the transcriptionally active form is trapped in an inactive complex with the inhibitory molecule IκB. Although in most cases the NF-κB DNA-binding activity is induced, in certain cell types, e.g. mature B cells, thymocytes, monocytes and macrophages, neurons, corneal keratocytes and vascular smooth muscle cells, it can be detected as a constitutively active, nuclear complex (Grilli *et al.*, 1993; Kopp and Ghosh, 1994). The basal transcriptional activity of NF-κB-binding sites was detected by a number of reporter experiments (Fan and Maniatis, 1989; Sif *et al.*, 1993). An increasing number

Fig. 6. Inhibition of NF-κB enhancer activity by NRF. Experimental outlines for (A), (B) and (E) are as in the legend of Figure 4A. (**A**) p0 reporter contains a CAT expression cassette. Reporters pP2G2, pP1IG2 and pSp1G2 contain two GAL4-binding sites 5′ close to the TATA-box. In pP2 and pP2G2, two copies of PRDII from the IFN-β promoter were integrated 5′ to the GAL4-binding sites of pG2 or 5′ to the TATA-box of p0. In pP1, pP1G2, pSP1 and pSP1G2, the PRDI element or the SP1-binding site was integrated into p0 and pG2, respectively. Co-transfected effector plasmids are indicated: the control expression plasmid is without an insert; pGAL4 expresses the GAL4 DBD; pNRF expresses full-length NRF; and pGAL4NRF codes for the N-terminal fusion of the GAL4 DBD to NRF. (**B**) In pL6P2, two copies of PRDII from the IFN-β promoter were integrated 3′ to the LexA-binding sites of pL6. Co-transfected effector plasmids are indicated: the control expression plasmid is without an insert; pLex expresses the LexA DBD; pNRF expresses full-length NRF; and pLexNRF codes for the N-terminal fusion of the LexA DBD to NRF. (**C**) Gel shift assay with ^{32}P-labeled double-stranded PRDII oligonucleotide alone (lane 6) or 10 µg of nuclear extracts prepared from untreated C243 cells (lanes 1–5). In lanes 1, 2, 3 and 4, the pre-formed complexes were incubated with 1 µg of αp50 (lane 1), αp52 (lane 2), αp65 (lane 3) or αc-Rel (lane 4) antibodies, respectively. In control lane 5, pre-formed complexes were incubated without antibodies. The unbound probe was run off the gel to facilitate resolution of the complexes. NF-κB complexes are indicated by closed triangles and the HMGI(Y) complex by the closed circle. (**D**) Co-transfected effector plasmids are indicated: pGAL4NRFdel1 encodes an N-terminal fusion of the GAL4 DBD to the deletion mutant of NRF lacking the NRE-binding domain (amino acids 1–296) and pGALNRFdel2 encodes an N-terminal fusion of the GAL4 DBD to the N-terminal domain of NRF (amino acids 1–193). Relative CAT expression is indicated in the diagram. The equal expression of the effector GAL4 fusion proteins was tested by Western blot analysis of 1 µg of nuclear extracts of the transfectants and GAL4-specific antibody (lower box). Double bands are due to proteolytic degradation in the GAL4 moiety. The shortened proteins are probably not able to bind to the GAL4 DBD. (**E**) In pP1P2G2, PRDI from the IFN-β promoter was integrated 5′ to PRDII of pP2G2. Experimental outlines are as described in Figure 4A and (A). Cells were mock induced (–) or induced by Sendai virus 24 h prior to the assay.
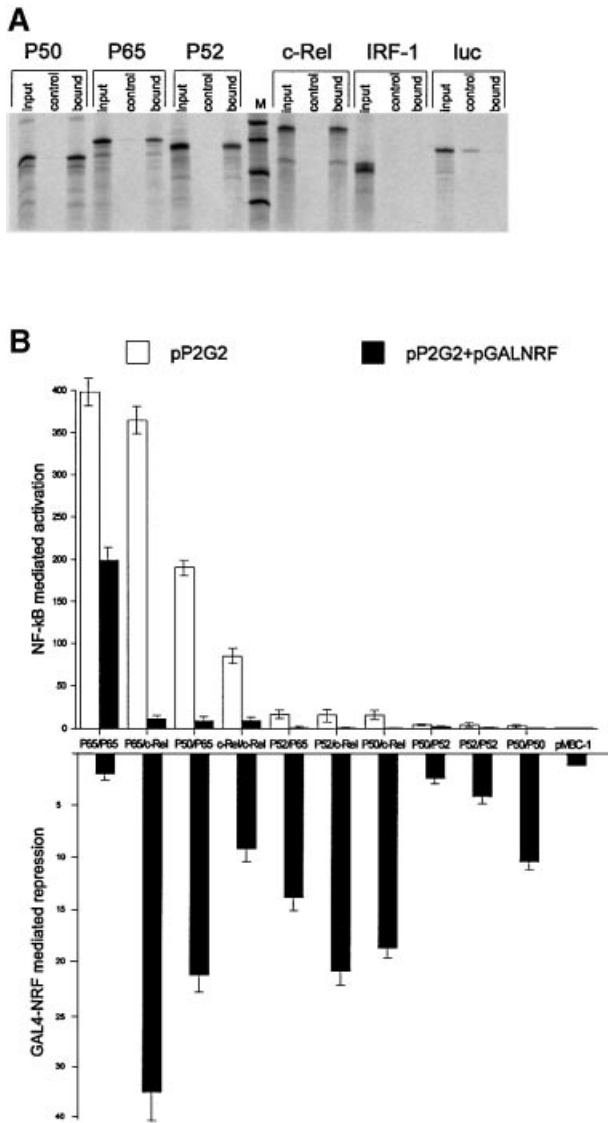
Fig. 7. Interaction of NRF with NF-κB factors. (**A**) Direct interaction of NRF with NF-κB factors *in vitro*. GST–NRF fusion protein was expressed in *E.coli*, purified and coupled to glutathione–Sepharose. Aliquots containing 100 ng of GST–NRF were incubated with ^{35}S-labeled NF-κB proteins, IRF-1 or luciferase after translation in rabbit reticulocyte lysate as indicated. Alternatively, labeled proteins were incubated with uncoupled glutathione–Sepharose as indicated (control). In comparison, 1/10 of the incubated amount of labeled proteins (input) and whole bound fractions are presented. The obtained signals were quantified and compared with the input amounts (data not shown). (**B**) Inhibition of NF-κB proteins by NRF. A 2 μg aliquot of a single (homodimer), 1 μg each of two (heterodimer) NF-κB expression plasmids or 2 μg of control plasmid without insert (pMBC-1) were transfected simultaneously with 1 μg of pP2G2 and 1 μg of pGALNRF or 1 μg of a control plasmid without insert. The reporter activity of pP2G2 by co-expression of pMBC-1 was adjusted to 1. Relative CAT activity was determined as described in Figure 4A. The reporter activities obtained from transfection experiments with or without NF-κB expression plasmids were compared in order to determine the NF-κB-mediated activation. The reporter activities obtained from transfection experiments with or without pGALNRF were compared in order to estimate the GAL4–NRF-mediated repression.

of reports revealed that IκB-independent repression mechanisms were involved in the NF-κB activity. For example, NF-kB-binding sites in the interleukin-6 (IL-6) and IL-8 genes are repressed directly by the glucocorticoid receptor

(Ray and Prefontaine, 1988; Mukaida *et al.*, 1994; Plaisance *et al.*, 1997). Recent studies have shown that the activated glucocorticoid receptor is capable of blocking the ability of NF-κB to activate gene transcription by an IκBα-independent pathway (Scheinmann *et al.*, 1995; Heck *et al.*, 1997; Linden *et al.*, 1997). A negative regulatory factor was identified that binds to the negative regulatory domain in the HIV-1 long terminal repeat (LTR) and inhibits the transcriptional activity of NF-kB-binding sites (Lu *et al.*, 1990; Hoover *et al.*, 1996).

The NRF-mediated silencing described here might be a general nuclear repression mechanism in other NF-κB-stimulated promoters. A number of NF-κB-driven promoters were found to be regulated in a fashion similar to that described in the IFN-β promoter. For example, by sequence comparisons, the promoters of HIV-1 (Hoover *et al.*, 1996), HTLV-1 (Tanimura *et al.*, 1993), IL-2 receptor α (Smith and Green, 1989) and IL-8 (Oliviera *et al.*, 1994) contain potential NRF-binding sites. Interestingly, the NRE-binding sites of the HTLV-I LTR and the IL-2 receptor α promoter are located in promoter regions which previously have been defined as negative regulatory domains (Smith and Green, 1989; Tanimura *et al.*, 1993). The NRE sequence was also found adjacent to the binding site of Dorsal, an NF-κB-homologous protein, in the *zen* promoter of *Drosophila*, indicating that the NRE sequence is evolutionarily conserved. The *Drosophila* protein DSP1 was identified by binding to this sequence (Lehming *et al.*, 1994). Detailed studies of DSP1 have shown that it disrupts the DNA binding of the TBP (TATA-box-binding protein) complex by direct binding to TBP, and acts as a transcriptional repressor for multiple activator families (Kirov *et al.*, 1996). Unlike DSP1, NRF does not decrease the basal transcriptional activity of the TATA-box, exemplified by the expression of NRF or LexA–NRF which do not decrease the basal activity of the TATA-box-directed transcription (see Figure 4). Furthermore, the SP1- or PRDI-directed transcription was not affected by NRF. We also find no homology between NRF and DSP-1 protein sequences. Therefore, it is unlikely that NRF is the human homolog of DSP1.

NRF seems to belong to a major class of transcriptional repressors which consist of a DNA-binding moiety and interact with other promoter elements to repress transcription via specific domains. Such transcriptional repressors have been termed active repressors because they act by direct protein–protein interaction and not by competitive mechanisms. Similarly to the activation domains, these repression domains are categorized loosely according to the primary amino acid sequence (Hanna-Rose and Hansen, 1996). However, we find no significant homology between the reported repression domains and the minimal repression domain in the NRF protein sequence.

A particular feature of the IFN-β promoter is that it is composed of regulatory elements, e.g. PRDI and PRDII, that can also be activated separately and individually by other signals in addition to virus infection. Synthetic promoters containing isolated copies of each of these elements display basal levels of activity and respond to several other inducers. In contrast, the IFN-β transcripts cannot be detected constitutively or in response to these signals. The most likely explanation for this discrepancy is a constitutive active repression mechanism in the native

IFN-β promoter. The antisense experiments presented here demonstrate the necessity for NRF for the constitutive repression of the endogenous promoter. Recent studies have shown that the highly specific activation of the IFN-β promoter is due to the fact that virus infection provides the only signal that can coordinate the action of all known IFN-β activators (Yie *et al*., 1999). This raises the question of how the NRF-mediated silencing in the IFN-β promoter is relieved by viral induction. No characteristics of the NRF protein, such as expression level, DNA-binding activity and nuclear localization, are altered by viral induction. Moreover, the silencing effect of the NRE on NF-κB sites, as demonstrated earlier (Nourbakhsh *et al.*, 1993), and the NRF-mediated repression on NF-κB proteins, as shown here, are also observed in virus-induced cells. It is therefore unlikely that the mode of NRF action is switched by virus-mediated signals. Uninfected cells contain very low nuclear amounts of IFN-β gene activators, but following virus infection these amounts are increased. However, our data show that the overexpression of NRF does not decrease the level of virus-induced activation of the IFN-β gene. Therefore, the inhibitory effect of NRF cannot simply be suppressed by the increased concentration of the IFN-β gene activators. This interpretation favors a model in which the action of NRF can be extinguished by the binding of a distinct set of virus-induced activator proteins to the promoter forming the IFN-β enhanceosome. Indeed, virus-induced synergy of PRDI (IRF-1-binding site) and PRDII (NF-κB-binding site) is sufficient to overcome the silencing effect of NRF. Recently, a detailed analysis of PRDI-binding factors *in vitro* revealed that the functional IFN-β enhanceosome can be assembled with either IRF-1 and IRF-3 plus IRF-7 or by IRF-7 alone (Yie *et al*., 1999). It was also shown that this synergistic activation requires a distinct domain in p65 (Merika *et al*., 1998). Interestingly, compared with the other NF-κB proteins, p65 is neither strongly inhibited nor bound by NRF (Figure 7A and B). It is therefore likely that in the virus-induced state, the p65-mediated synergy and subsequent formation of the IFN-β enhanceosome bypasses the inhibitory function of NRF.

Besides the physiological relevance of NRF due to the constitutive silencing of the IFN-β gene, an important feature of NRF is that it interacts directly with the NF-κB proteins by an active repression mechanism. As demonstrated by the GAL4–NRF or LexA–NRF fusion proteins, NRF contains a separable and position-independent repression domain. In principle, this portable NF-κB-repressing domain can be recruited to another target promoter via a suitable DNA-binding domain. Although this has to be confirmed experimentally, the use of such a fusion protein could be an attractive approach for attenuation of the NF-κB activity in its target promoters.

Materials and methods

South-Western analysis
South-Western analysis according to standard protocols (Sambrook *et al*., 1989) using ^{32}P-labeled double-stranded oligonucleotides which contain the single NRE sequence 5′-aattcgAATTCCTCTGAcgaaca-3′ proved to be the optimal method. Nuclear extracts from HeLa cells were prepared according to Dignam *et al*. (1983) with modifications introduced by

Wildeman *et al*. (1984). Bacterial extracts from the Y9010 strain were prepared by sonification and sedimentation of cell debris.

Cloning the NRF cDNA
A Uni-ZAPTMXR cDNA library (Stratagene) from HeLaS3 cells was screened according to Singh *et al*. (1988) and Vinson *et al*. (1988) using the same ^{32}P-labeled double-stranded oligonucleotide as applied for Western blotting. Bacterial DNA served as unspecific competitor. Positive plaques were isolated and tested repeatedly for specific and unspecific binding. An oligonucleotide containing the mutant NRE with the sequence 5′-aattcgAATTCCCCCGAcgaaca-3′ was used to eliminate unspecifically binding clones. cDNA inserts were isolated by *in vivo* excision from the pBLUESCRIPT phagemid from the λ vector according to the manufacturer's instructions (Stratagene). The nucleotide sequence of the complete cDNA was obtained by PCR using primer sets complementary to the 5′ end of the insert and 5′ to the cloning sites of the λ library.

Northern blotting and hybridization
Human multiple tissue Northern (MTN) blot I and II were obtained from Clontech (7760-1 and 7759-1). Northern blot analysis was performed according to Sambrook *et al*. (1989). The hybridization procedure was carried out according to the manufacturer's protocol.

Expression of GST fusion protein in Escherichia coli
The NRF coding sequence was integrated into pGEX-2TK (Pharmacia) and transformed into the *E.coli* BL21 strain for expression of GST–NRF fusion protein. Transformation, isopropyl-β-D-thiogalactopyranoside (IPTG) induction and purification of fusion proteins were performed according to the manufacturer's instructions (Pharmacia).

Expression plasmids
Reporter plasmids. Plasmid pG2 containing two copies of the GAL4-binding site and pL6 containing six copies of the LexA-binding site was kindly provided by K.Chow (Weintraub *et al*., 1995). GAL4-binding sites were removed to obtain p0. Dimeric PRDII, PRDI and NRE sequences described earlier (Nourbakhsh *et al*., 1993) and a single SP1-binding site were integrated as synthetic double-stranded oligonucleotides into the *Sph*I site of pG2 or p0 to give pP1P2G2, pP2G2, pP2, pP1, pP1G2, pSP1, pSP1G2 pNm and pN. The sequences of the integrated oligonucleotides are as follows: P2, 5′-ccatggGTGGGAAATTCC-GTGGGAAATTCCcatg-3′; P1, 5′-ccatggAAGTGAAAGTGAAAGTG-Acatg-3′; SP, 5′-ccatggGGGAGGCGTGGCCTGGGCGGGACTGGGG-AGTGCcatg-3′; N, 5′-ccatggAATTCCTCTGAcatg-3′; and Nm, 5′-cc-atggAATTCCCCCGAcatg-3′.

Effector protein expression plasmids. Plasmids containing the GAL4 and LexA DBDs and the VP16 activation domain were kindly provided by K.Chow and P.Broad. All coding sequences were integrated into pMBC-1 (Dirks *et al*., 1994) to construct pGAL4, pVP16, pGAL4NRF, pG-AL4NRFdel1, pGAL4NRFdel2, pNRFVP16, pDBDVP16 and pNRF driven by the MT7 promoter which is not affected by NF-κB proteins.

NF-κB expression plasmids. Coding sequences for p50, p52, -p65 and c-Rel were kindly provided by K.Scheidereit. These coding sequences were amplified by PCR and integrated into the poly linker of pMBC-1 to ensure equal expression of NF-κB proteins.

Cell lines and DNA transfection
C243 (Oie *et al*., 1977) or LMTK (ATCC, CCL-1.3) cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin were transfected by calcium phosphate co-precipitation. A total of 10 μg of DNA containing 2 μg of reporter plasmid, 0.5 μg of luciferase expression plasmid, 4 μg of expression plasmid encoding the effector protein and a suitable complementing amount of empty expression vector per 1.5×10^5 cells was transfected (Sabe *et al*., 1984). After 72 h, cells were harvested and pooled for reporter gene analysis.

C243-tTA cells constitutively expressing the synthetic transactivator tTA were kindly provided by S.Kirchhoff (Kirchhoff *et al*., 1995); they were maintained in medium as described above supplemented with 2 μg/ml tetracycline and 500 μg/ml G418, and were transfected by calcium phosphate co-precipitation. Expression plasmid ptTA (5 μg) (Gossen and Bujard, 1992) containing sense or antisense NRF, 10 μg of high molecular weight DNA from LMTK cells and 0.5 μg of selection plasmid pSV2PAC (de la Luna *et al*., 1988) containing the puromycin acetyltransferase gene per 2×10^5 cells were transfected. More than 100 puromycin-resistant clones were pooled and tested for

interferon and NRF expression. Human interferon was measured using the antiviral assay on Vero cells and vesicular stomatitis virus (VSV) as challenging virus as described earlier (Nourbakhsh *et al*., 1993).

Gene expression analysis and induction protocols

Transiently transfected cells were washed in phosphate-buffered saline (PBS) and pelleted at 250 *g*. Cell pellets were resuspended in 250 mM Tris (pH 7.5) and cell lysates were prepared by freezing and thawing. The luciferase assay was performed according to Williams *et al*. (1989), and CAT expression was analyzed using the CAT ELISA kit (Boehringer Mannheim). The results were normalized to luciferase activity and protein content.

For induction, 10^6 cells per ml and cm^2 were plated and induced after 24 h with Sendai virus or poly(I):poly(c) or mock induced. Induction was carried out by the addition of 10 p.f.u. of Sendai virus per cell in serum-free DMEM for 1 h. Following induction, the cells were washed and incubated in fresh DMEM plus 10% fetal calf serum for 18 h for the interferon assays or for the indicated times following mRNA preparations. Murine interferon was measured using the antiviral assay on LMTK cells and VSV as a challenging virus. Trizol reagent (Gibco-BRL, Life Technologies) was used for the isolation of total RNA from cells according to the manufacturer's instructions. Poly(A) RNA was isolated from total RNA using Dynabeads Oligo (dT)25 (Dynal) according to the manufacturer's instructions.

The expression of GAL4–NRF fusion proteins was measured by Western blot analysis as described by Harlow and Lane (1988) using 1 µg of nuclear extract and polyclonal antibody directed against the GAL4 DBD obtained from Santa Cruz. The endogenous NRF protein was detected by immunoprecipitation using an equal mix of polyclonal antibodies directed against amino acids 256–272 and 272–288 (see below) and protein A/G–Sepharose obtained from Santa Cruz according to the manufacturer's instructions. Following immunoprecipitation, cleared extracts were analyzed by Western blot using a mix of polyclonal antibodies directed against amino acids 25–45,175–191 and 364–382 of NRF.

Preparation of extracts and gel retardation assays

Nuclear extracts from HeLa cells were prepared according to Dignam *et al*. (1983) with modifications introduced by Wildeman *et al*. (1984). Crude extracts from *E.coli* were treated according to the protocol provided with the GST expression kit (Pharmacia). Gel shift analysis was carried out according to the protocol of Fried and Crothers (1981) with the following modifications: the indicated amounts of protein were incubated with 3 fmol (20 000 c.p.m.) of labeled double-stranded oligonucleotide in the presence of 0.01 U of poly(dI:dC) in 10 mM HEPES pH 8.0, 5 mM $MgCl_2$,50 mM KCl, 0.025% bromophenol blue, 0.025% xylene cyanol and 10% Ficoll for 10 min at room temperature. The samples were loaded on pre-electrophoresed 8% native poly-acrylamide gels. The gels were run for at least 8 h at 100 V. After drying, the gels were analyzed using a Molecular Dynamics 400A PhosphoImager System.

In vitro protein–protein interaction assays

The radioactively labeled NF-κB proteins were synthesized using the TNT *in vitro* translation system obtained from Promega according to the manufacturer's instructions. NRF interactions were carried out by using the GST–NRF fusion protein expressed in and purified from *E.coli* strain BL21. In each reaction, 1 µg of purified GST–NRF was immobilized on glutathione–Sepharose. After incubation of 50 ng of ^{35}S-labeled NF-κB proteins at 30°C for 1 h in PBS with 100 µg of bovine serum albumin, the beads were washed four times with 500 µl of incubation buffer (140 mM NaCl, 2.68 mM KCl, 4.3 mM NaH_2PO_4, 1.47 mM KH_2PO_4). Bound proteins were eluted with SDS loading buffer and analyzed with the inputs by SDS–PAGE. After drying, the gels were analyzed using a Molecular Dynamics 400A PhosphoImager System.

Preparation of NRE-specific antisera

Based on the antigenic index of the NRF protein sequence, we selected five short peptide sequences corresponding to amino acids 25–45, 175–191, 256–272, 272–288 and 364–382. These peptides were each injected into rabbits. The indicated amounts of an equal mixture of the obtained antisera (see figure legends) were used for the identification of the human and murine NRF in Western blot analysis, by immunoprecipitation or in supershift experiments. An equal mixture of pre-immune sera was also used for each control experiment.

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