Identification of distal silencing elements in the murine interferon-A11 gene promoter

Pascal ROFFET*, Sébastien LOPEZ*, Sébastien NAVARRO, Marie-Thérèse BANDU, Colette COULOMBEL, Madeleine VIGNAL, Janine DOLY and Guilane VODJDANI†

Laboratoire de Régulation de l'Expression des Gènes Eucaryotes, UPR 37-CNRS, UFR Biomédicale, Université René Descartes, 45, Rue des Saints-Pères, 75270 Paris Cedex 06, France

The murine interferon-A11 (Mu IFN-A11) gene is a member of the IFN-A multigenic family. In mouse L929 cells, the weak response of the gene's promoter to viral induction is due to a combination of both a point mutation in the virus responsive element (VRE) and the presence of negatively regulating sequences surrounding the VRE. In the distal part of the promoter, the negatively acting E1E2 sequence was delimited. This sequence displays an inhibitory effect in either orientation or position on the inducibility of a virus-responsive heterologous promoter. It selectively represses VRE-dependent transcription but is not able to reduce the transcriptional activity of a VRE-lacking promoter. In a transient transfection assay, an E1E2-containing DNA competitor was able to derepress the native Mu IFN-A11 promoter. Specific nuclear factors bind to this sequence; thus the binding of *trans*-regulators participates in the repression of the Mu IFN-A11 gene. The E1E2 sequence contains an IFN regulatory factor (IRF)-binding site. Recombinant IRF2 binds this sequence and anti-IRF2 antibodies supershift a major complex formed with nuclear extracts. The protein composing the complex is 50 kDa in size, indicating the presence of IRF2 or antigenically related proteins in the complex. The Mu IFN-A11 gene is the first example within the murine IFN-A family, in which a distal promoter element has been identified that can negatively modulate the transcriptional response to viral induction.

INTRODUCTION

Eukaryotic gene expression is regulated primarily at the transcriptional level and involves interaction of specific *trans*-regulator proteins with particular target DNA *cis*-elements present mainly in the promoter. The expression patterns are the consequence of the combination of both positive and negative control. Several mechanisms have been proposed to explain how transcriptional repression occurs [1–4]. The repressor can for instance inhibit the DNA binding of a particular activator by competing for a common binding site (steric occlusion) or by interacting with the DNA binding domain of the activator (squelching). The negative factor can also interact with the activating domain of the positive factor and inhibit its effect (quenching). The last mechanism is the silencing or 'direct repression' of the basal transcriptional machinery independent of the presence of positive elements. In this case the repressor binds to a silencer sequence and displays its negative effect in either orientation or position.

The interferon (IFN) system has been widely studied as a model for understanding the mechanisms of eukaryotic gene regulation. After stimulation by virus, type-I IFN (IFN-A and -B) is expressed as a result of transcriptional activation [5,6]. This response is a transient phenomenon leading to an accumulation of high levels of IFN mRNA [7] followed by an efficient turnoff which is due to a combination of transcriptional repression [8] and rapid turnover of the mRNA [6,9]. The mechanisms by which IFN gene promoters are activated upon induction have been most particularly studied within the human IFN-B promoter [10]. It is now accepted that several overlapping positive regulatory domains (PRDI, II, III, IV) and negative regulatory domains (NRDI, II) [10–12] encompassing the IRE (IFN gene regulatory element) are involved in the regulation of the human IFN-B gene expression in response to induction [13]. All of these elements are binding sites for specific nuclear factors [14–16]. Nuclear factor (NF)-κB is activated by virus induction and participates in the transcriptional activation of the IFN-B gene by binding to the PRDII motif [16–19]. Activating transcription factor-2 binds to PRDIV and has been shown to be involved in virus induction [20]. Moreover, the binding of high-mobility group I(Y) proteins to PRDII and PRDIV apparently alters the DNA structure thereby increasing the binding activity of both NF-κB and activating transcription factor-2 [21]. IFN regulatory factors, IRF1 and IRF2 [14,22–24], bind to PRDI and PRDIII. After virus induction, it was originally reported that IRF1 stimulated transcription from the IFN-B promoter while IRF2 antagonized this activation by competing for the same DNA-binding site [23]. Studies on targeted disruption of the IRF1 gene in mice or embryonic stem cells remain controversial as to the role of IRF1 in the viral induction of type-I IFN genes, while the repressing effect of IRF2 appears confirmed in IRF2-deficient mice [25–28]. Concerning the negative elements, the negative regulatory element (NRE) sequence partially overlaps PRDII and is the binding site for negative factors [29] including DSP-1, a *Drosophila* high-mobility group 1-like protein [30]. Virus induction can overcome the silencing activity of the NRE by the co-operative effect of PRDI and PRDII. The cDNA encoding a

Abbreviations used: IFN, interferon; VRE, virus responsive element; IRE, IFN gene regulatory element; PRD, positive regulatory domain; NRD, negative regulatory domain; NRE, negative regulatory element; IRF, IFN regulatory factor; NDV, Newcastle disease virus; CAT, chloramphenicol acetyltransferase; IE, inducible element; Mu, murine; Hu, human; NF, nuclear factor.

* Should be considered as equal first authors.

† To whom correspondence should be addressed.

PRDI-binding protein, PRDI-BF1, which acts as a post-induction repressor of the IFN-B gene, has also been identified [8].

The transcriptional regulation of IFN-A genes has been less studied and very little is known about the factors binding to different elements in the virus-responsive element (VRE) [31]. In the VRE ($-109/-64$) of the human (Hu) and murine (Mu) IFN-A gene promoters, homologies to PRDI and PRDIII have been found. Transcriptional control of IFN-A genes might thus involve some factors in common with the IFN-B, as observed in a number of reports [31–34]. However, some other evidence indicates that the IFN-A and -B genes are transcriptionally activated through different mechanisms [31,34–37]. A 'TG protein' has been reported to bind to the TG sequence in the Hu VRE-A1 and not to Hu IFN-B PRDI nor PRDII; it might be a specific IFN-A *trans*-activator [34]. It has been reported that overexpression of IRF1 was able, independently of virus induction, to activate transcription from chimaeric promoters containing the VRE from the Hu IFN-A1 [34]. Activation was also observed with the inducible element (IE) ($-109/-75$) from the Mu IFN-A4 and -A6 genes [38] in transient assays; yet the endogenous Mu IFN-A genes could not be induced by this overexpression. A newly identified factor, αF1/B, binds to the IE in the Mu IFN-A4 promoter [39]. This factor is not a member of the IRF family and it has been hypothesized [38,39] that it might co-operate with IRF1 for an efficient activation through the IE sequence. To date, none of these factors has yet been isolated or characterized. Recent studies on Mu IFN-A gene activation indicate that a multicomponent virus-induced factor (VIF), different from the previously described factors and from IRF1, binds to the IE and may participate in transcriptional activation upon virus induction [40].

IFN-A genes are members of a multigenic family and exhibit differential expression patterns in a cell-type and inducer-specific manner in both murine [41–47] and human [35] systems. Also, for a given cell type and a given inducer, the level of expression of each member of the multigenic family differs. In this respect, we have previously shown that the Mu IFN-A11 gene [47,48] is poorly expressed upon Newcastle Disease Virus (NDV) induction in L929 cells, whereas Mu IFN-A4 is highly inducible. Thereby, the Mu IFN-A11 gene is considered as a repressed gene. Our previous work has established that the weak response of the Mu IFN-A11 gene to NDV in L929 cells is due in part to the presence of negatively acting regions in the distal promoter and in part to a crucial point mutation in the VRE of Mu IFN-A11 (VRE11) as compared with the VRE4 [48]. The removal of the upstream negative domain (-470 to -145) increases virus-inducibility of the Mu IFN-A11 gene. We therefore attempted to define precisely the boundaries of the negative *cis*-acting sequences in the distal regions of the Mu IFN-A11 promoter. In this report we present a detailed analysis of the E1E2 sequence which displays a negative effect on virus-induced expression from an inducible VRE-containing heterologous promoter in an orientation- and position-independent fashion, but has no effect on a constitutive heterologous promoter. In the native context, deletion of this sequence derepresses the activity of the IFN-A11 promoter. Our results show that the binding of *trans*-regulators participates in the repression of the Mu IFN-A11 gene. The E1E2 segment contains several sequence features such as a tandem repeat, two palindromes and an IRF-binding site. Recombinant IRF2 bound this sequence and anti-IRF2 antibodies supershifted a major complex formed with nuclear extracts. This complex contains a 50 kDa protein similar in size to IRF2, thus indicating the presence of IRF2 or antigenically related proteins in the complex. The role of IRF2 and the mechanisms of repression accounting for the negative effect of E1E2 are discussed.

MATERIALS AND METHODS

Oligonucleotides

Double-stranded DNA oligomers were obtained by annealing chemically synthesized complementary strands. The 47-bp B and 22-bp C oligomers are blunt-ended double-stranded DNAs corresponding, respectively, to the sequences from positions -260 to -214 and -213 to -192 of the Mu IFN-A11 upstream region (see Figure 3B). The 46-bp E1E2, 23-bp E2 and 20-bp delta E1E2 are double-stranded oligonucleotides corresponding, respectively, to the sequences from positions -244 to -199, -222 to -199 (see Figure 3B) and -199 to -180. The 24-bp $(AAGTGA)_3$ oligonucleotide is a high-affinity IRF-binding site and corresponds to the 5′-TCG$(AAGTGA)_3$GAC-3′ DNA molecule. EKSB is the DNA oligonucleotide which was used as a non-relevant competitor and consists of an 18-bp *Eco*RI–*Kpn*I–*Sac*I–*Bam*HI polylinker. The 69-bp BC segment was obtained using single-stranded B and C oligonucleotides as primers in a PCR.

Construction of reporter plasmids

The pABCD-VRE4*tk* series of constructions (Figure 1) was obtained by using the pVRE4 plasmid [48] which carries the synthetic Mu IFN-A4 promoter region corresponding to the VRE-A4, flanked on either side by *Hin*dIII and *Bam*HI linkers and inserted at the *Hin*dIII and *Bam*HI sites in pBLCAT2 [49]. The pABCD-VRE4*tk* series was constructed either by subcloning blunt-ended restriction fragments ($-470 = Dde$I; $-261 = Hin$fI; $-213 = Dra$I; $-192 = Alu$I; $-147 = Bst$XI) of the Mu IFN-A11 promoter [47] or by cloning the oligonucleotides corresponding to the B, C, E2 and E1E2 fragments into the unique blunt-ended *Hin*dIII site of pVRE4. The VRE4–*tk*–E1E2 is obtained by cloning E1E2 oligonucleotide into the *Sma*I site of pVRE4. The pE1E2–*tk* was obtained by cloning the E1E2 into the *Sal*I blunt site of pBLCAT2. The pIF11T and p($-119/+19$)-CAT plasmids corresponding, respectively, to the $-470/+19$ and $-119/+19$ promoters of Mu IFN-A11 inserted in pBLCAT3 [49] have been described previously [48]. The 5′ deletions of the Mu IFN-A11 promoter fragments were obtained by PCR using E1E2 for p($-244/+19$)-CAT and delta E1E2 for p($-199/+19$)-CAT as 5′ primer and for both constructions the *Bam*HI/A+19 oligonucleotide (5′-CCCAGATCTGGATCC-TCT-3′) as 3′ primer. All fragments were inserted in *Xba*I blunt/*Bam*HI sites of pBLCAT3. All constructions were checked by nucleotide sequencing on double-stranded DNA [50].

DNA transfection, viral induction and enzymic assays

L929 cells (3.5-cm-diam. dishes), seeded in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum, were transfected at 50% confluency by the calcium phosphate precipitation method [51] with 1.25 μg of reporter plasmid in the presence of 125 ng of pCMV-βGal or 250 ng of pEF-LacZ plasmids (kindly provided by Dr. D. Biard, CEA, Fontenay-Aux-Roses, France, and Dr. J. Mushler, Institut Pasteur, Paris, France, respectively), and followed 4 h later by a 1 min glycerol (10%) shock. NDV induction was carried out 48 h later as described [48]. In the mock-induced conditions only NDV addition was omitted. Cells were harvested 24 h post-induction and cytoplasmic extracts were prepared by cell lysis with a buffer containing 0.65% Nonidet P-40, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5 mM PMSF (Dr. M. Parker, personal communication). Chloramphenicol acetyltransferase (CAT) assay was carried out as described [52]. Transfection

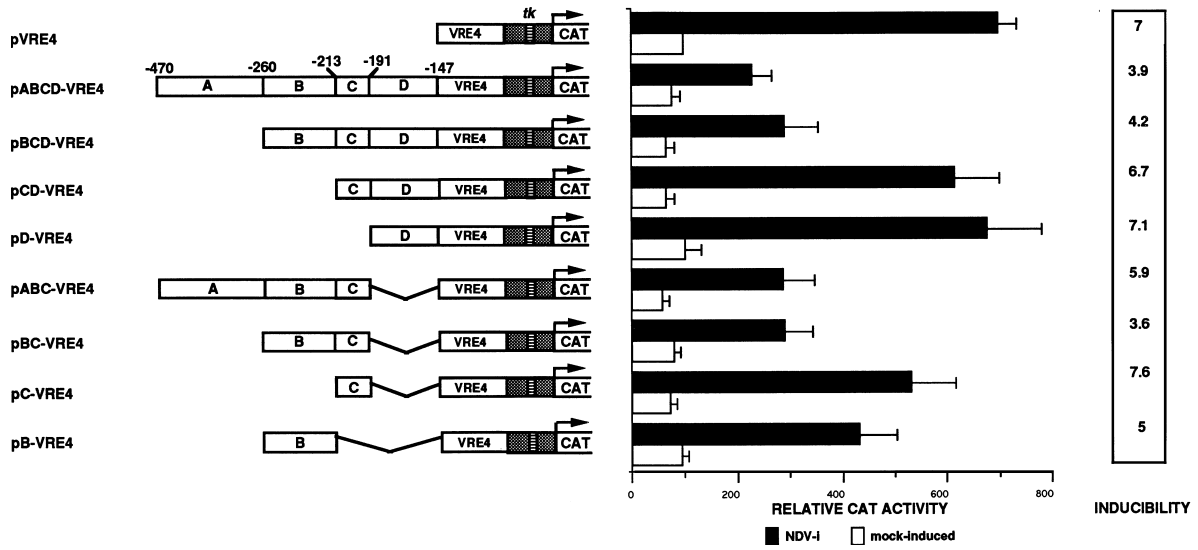

Figure 1 The BC segment from the Mu IFN-A11 gene upstream sequence has a negative effect on the VRE4-*tk* promoter activity

The segments have been named: A from −470 to −261, B from −260 to −214, C from −213 to −192 and D from −191 to −147. The name and structure of the different chimeric plasmids tested are presented in the left-hand panel. L929 cells were transfected and induced as described in the Materials and methods section. In cell lysates, CAT assays were normalized to β-galactosidase activity. Histogram bars in the right-hand panel represent CAT activities ± S.E.M. for at least five separate transfections relative to the mock-induced activity of pVRE4 which was set at 100%. Inducibility is the ratio of the NDV-induced activity over the mock-induced activity. With the pABCD-, pBCD-, pABC- and pBC- constructs, induced CAT activities in each experiment were always, or with pB- most often, significantly different from the reference pVRE4 plasmid ($P < 0.01$), whereas pCD-, pD-VRE4 and pC-VRE4 were not.

efficiency was determined by the β-galactosidase activity assay with the Galactolight™ (Tropix, Inc.) chemiluminescence kit. In each experiment, a given construction was transfected in duplicate and two different clones of each construction were tested. The significance of pairwise comparisons between a given construction and pVRE4 reference was determined by Student's *t*-test.

Ex vivo transfection-competition experiments

For *ex vivo* transfection-competition experiments using oligonucleotides as competitor [53], L929 cells were co-transfected as described above, either with pBCD-VRE4*tk* or pIF11T reporter plasmids, 125 ng of the pCMV-βGal normalizing plasmid and different fold molar excesses of competitor oligonucleotides BC, B, C and EKSB. The pUC18 plasmid was used as carrier to complete each DNA mixture to 2.5 μg. Enzymic activity was monitored with extracts from NDV-induced or mock-induced L929 cells as described above.

DNA binding analysis

Nuclear extracts from NDV-induced (8 h) and uninduced L929 cells were prepared as described [54]. Gel shift assays with recombinant Hu IRF2 protein (kindly provided by Dr. J. Hiscott, McGill University, Montreal, Quebec, Canada) were performed as described [55]. Complexes were resolved by electrophoresis on 5% native polyacrylamide gels. Supershift assays using human (kindly provided by Dr. J. Hiscott) and murine (kindly provided by Dr. T. Taniguchi, University of Tokyo, Tokyo, Japan) anti-IRF2 and anti-IRF1 antibodies were performed as described elsewhere [55]. For the DNase I footprinting assays, the BCD fragment in the pBCD-*tk* plasmid was used after appropriate enzymic digestion to label either the coding or the non-coding strands. DNase-I footprinting experiments were carried out as described in the SureTrack protocol (Pharmacia). Depending on the probe used, the samples were analysed on a 6 or 8% sequencing gel.

UV cross-linking

UV cross-linking of nuclear proteins to the E2C DNA was performed as described previously [56]. E2C is obtained by annealing the following oligonucleotides: the E2 coding and the C non-coding strands, thus generating 5′ and 3′ overhangs. The duplex molecules were blunt-ended by Klenow filling using BrdU and labelled [α-^{32}P]dATP and [α-^{32}P]dGTP nucleotides.

RESULTS

NREs in the upstream region of the Mu IFN-A11 gene repress a VRE-containing promoter

The weak response of the Mu IFN-A11 promoter to virus induction in L929 cells was shown to be due to both the presence of negatively acting regions surrounding the VRE and a nucleotide substitution in the VRE which is responsible for the moderate response of the VRE11, as compared with the VRE4, to viral induction in a heterologous promoter [48]. To determine the sequences of the Mu IFN-A11 upstream region that prevent the response of the VRE to virus induction, hybrid plasmids carrying various lengths of the gene promoter were constructed. The different 5′- and 3′-deleted fragments were generated from the −470 to −147 ABCD promoter region. They were inserted in the pVRE4 plasmid, upstream of the VRE4 oligonucleotide which is placed 5′ to the *Herpes simplex* thymidine kinase (*tk*) gene promoter controlling the expression of the bacterial CAT reporter gene. Therefore, the VRE4 renders the promoter virus inducible. L929 cells were co-transfected transiently with these

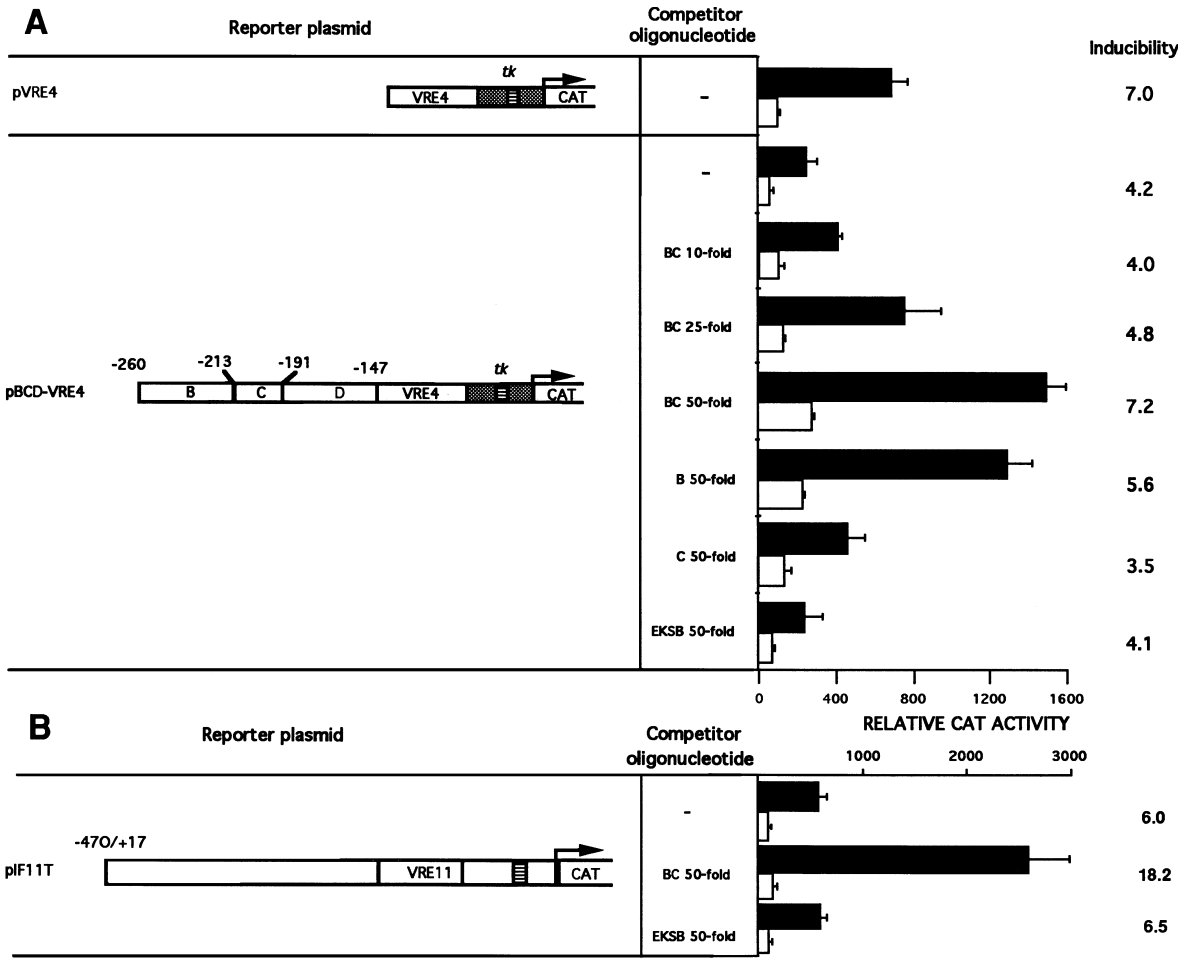

Figure 2 Protein binding to the BC segment is responsible for the negative effect on promoter activity

Ex vivo transfection-competition assays were carried out as described in the Materials and methods section. The reference plasmid and the constructions to be derepressed are depicted in the left-hand panel. In the right-hand panel, relative CAT values are represented either by black (NDV-induced) or open (mock-induced) histogram bars. (**A**) Derepression of the chimaeric promoter. L929 cells were co-transfected with the pBCD-VRE4 plasmid and 10-, 25- and 50-fold molar excesses of BC or 50-fold molar excess of B or C oligonucleotides and completed to the same amount of DNA with pUC18. The non-specific competitor DNA was the EKSB oligonucleotide. The 100% corresponds to the mock-induced CAT activity of pVRE4. (**B**) Derepression of the Mu IFN-A11 native promoter. The pIF11T plasmid was co-transfected with the BC or the non-relevant EKSB oligonucleotide (50-fold molar excess). CAT activities $\pm$ S.E.M. for the four separate transfections are expressed relative to the mock-induced level of pIF11T taken as 100%.

constructs together with a β-galactosidase-expressing vector, and then they were either NDV- or mock-induced.

The results of transient-transfection experiments are summarized in Figure 1. As the data are pooled from several experiments, they are presented in arbitrary units of CAT activity, with 100% corresponding to the mock-induced activity of pVRE4. After NDV induction, the pABCD-VRE4 plasmid shows a 67% reduction of CAT activity when compared with the pVRE4 plasmid. As was observed in the native promoter [48], these results confirm, in a chimaeric system, the presence in the distal region of the IFN-A11 promoter of a negatively acting domain. With the 5′ and 3′ deletions of the ABCD segment, one can observe that the plasmid constructions bearing the B and C fragments together display the most marked effect in reducing the NDV-induced response of the pVRE4. It can be pointed out that in the constructions containing the B segment, the inducibility decreases. This is due to the fact that the transcriptional activity is less reduced in the mock- than in the NDV-induced conditions. The C and D segments did not modify the virus inducibility. The B and C fragments, alone, are not able to display the same reduction as the joint BC fragment on the virus-activated transcription of the promoter, suggesting that either B and C fragments co-operate for the repressing activity or the cleavage between B and C fragments disrupts an element involved in the repression. Therefore, it seems that the Mu IFN-A11 promoter domain between -260 and -192 contains a core fragment with negative regulatory activity.

Competition analyses demonstrate a functional interaction of the BC segment with *trans*-repressor(s)

To investigate whether the negative effect observed on promoter activity is due to *trans*-repressor binding to the BC segment, *ex vivo* transfection-competition analyses were carried out by co-transfecting L929 cells with an excess of the BC fragment with either the chimaeric or the IFN-A11 native promoter constructions. If the cellular concentration of the *trans*-acting factor(s) is limiting, its transcription regulatory effect will be partially titrated out by co-transfection with the BC fragment.

In the experiments shown in Figure 2(A), increasing amounts

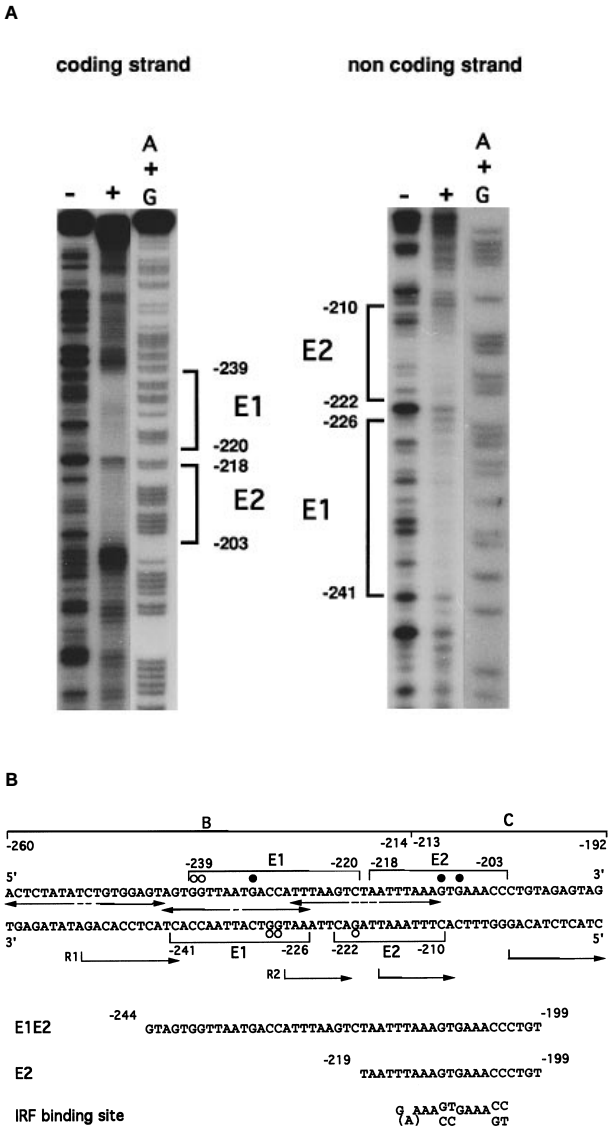

Figure 3 Localization of nuclear protein binding activities within the BC fragment

(**A**) DNase I footprinting analysis in the coding or non-coding strand on the BCD probe was performed as described in the Materials and methods section. Lane (−) shows the DNase I pattern obtained with the probe in the presence of BSA; lane (+) corresponds to the pattern in the presence of nuclear extracts from uninduced L929 cells; lane A + G corresponds to the products of purine-specific chemical cleavage. Numbers in the centre indicate the nucleotide distance of the protected regions from the Mu IFN-A11 cap site. E1E2 is the name given to the protected region. (**B**) Summary of DNase I footprinting analysis. The nucleotide sequence of the coding and non-coding strands of the Mu IFN-A11 BC fragment is shown. The delimitations of the B and C fragments, as well as those for the protected E1E2 region, are indicated by brackets. Numbering is relative to the transcription initiation site of each promoter. The palindromic sequences and the direct repeats (R1 and R2) are denoted by arrows. From methylation interference assays, the G residues which interfere weakly (open circles) or strongly (closed circles) are indicated. The sequences at the bottom correspond to E1E2, E2 oligonucleotides and the consensus sequence for the IRF-binding site.

of oligonucleotide BC were able to gradually relieve the transcriptional repression exerted by BC in the chimaeric construct, in a dose-dependent fashion. Competition with the BC and B oligonucleotides in a 50-fold molar excess restores totally (7.2-fold) or partially (5.6-fold) the virus inducibility of the pBCD-VRE4 plasmid to that of the pVRE4 reference. Although the C oligonucleotide slightly increases the level of transcription it does not restore inducibility. These results accord well with the corresponding behaviour of each fragment in the transient-transfection experiments described above. Competition with a non-relevant oligonucleotide did not modify the inducibility of the pBCD-VRE4 plasmid. It may be pointed out that with molar excesses of BC or B oligonucleotides, the levels of CAT activity in the induced and mock-induced conditions exceed those of pVRE4, suggesting that competition with the BC segment not only restores the VRE4 responsiveness to viral induction but also relieves a constitutive level of repression of the pBCD-VRE4 plasmid. As a control, co-transfection experiments were performed with the test pBCD-VRE4 plasmid using as competitors a 50-fold molar excess of pBCD-*tk*Δ or pBC-*tk*Δ plasmids in which a *tk*-CAT portion was deleted. We obtained a derepression similar to that described above (results not shown).

We also tested whether the weakly inducible pIF11T plasmid [48] containing the native promoter of the Mu IFN-A11 gene (−470/+17) could be derepressed by competition with the BC oligonucleotide. Co-transfection of L929 cells with pIF11T and a 50-fold molar excess of competitor yielded an increase in the level of CAT activity and in the virus inducibility of pIF11T (Figure 2B).

These results allow the conclusion that the BC fragment exerts its transcription-repressing activity by the binding of one or more *trans*-acting protein factors present in limiting concentrations in L929 cells.

E1E2 is the binding site for nuclear protein(s) within the BC segment

To determine the recognition sequences for the protein(s) binding to the BC segment, we performed DNase I footprinting analysis with nuclear extracts from either NDV-induced or uninduced L929 cells. As shown in Figure 3(A), protein binding protects a minimal E1E2 region subdivided into two subregions that we have defined as E1 and E2. The E1 subregion corresponds to a 20-bp (−239/−220) and a 16-bp (−241/−226) protected region on the coding and non-coding strands respectively and its pattern covers a palindrome (Figure 3B). The E2 subregion is located between −218 and −203 (16 bp) in the coding strand and between −222 and −210 (13 bp) in the non-coding strand. The first noticeable point with the E2 subregion is that it actually overlaps the arbitrary B and C junction, explaining the results in the transient-transfection assays where the B and C segments have shown the importance of being associated for an optimal negative effect. Furthermore, the protected region in the non-coding strand partially overlaps both a tandem repeat (8/9 nucleotide homology) and another palindrome from position −228 to −209. Two other direct repeated sequences (10/11) flank exactly the E1E2 footprint pattern. DNase I footprinting assays were performed in the presence of either uninduced or NDV-induced nuclear extracts using the BCD-VRE4 fragment from the chimaeric promoter or the −260/+19 native Mu IFN-A11 promoter in comparison with the BCD segment alone to check any possible modification of the E1E2 pattern due to the presence of the VRE or to nuclear extracts. No difference in the E1E2 protected pattern was detected in either case nor on any strand. We further performed DNA methylation interference analyses to identify in the BC segment the guanosine residues involved in the essential contacts when factors bind to the E1E2 sequence. The G residues which are contact points for protein–DNA complexes are localized in the E1E2 footprint pattern and are indicated in Figure 3(B).

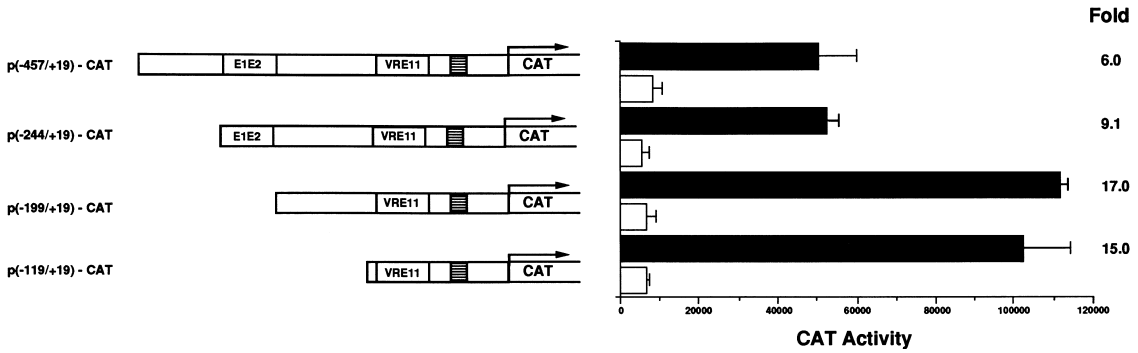

Figure 4 Deletion of the E1E2 sequence derepresses the Mu IFN-A11 native promoter

The names and structures of the different plasmids are presented in the left-hand panel. Assay conditions are as described in the legend to Figure 1. The histogram bars show a representative assay. Error bars indicate S.E.M.s of duplicate samples. At least three independent experiments yielded essentially identical results (variations $< 15\%$). Pairwise comparisons between p(−244/+19)-CAT or p(−457/+19)-CAT constructs and p(−199/+19)-CAT or p(−244/+19)-CAT are significantly different with $P < 0.01$ (Student's *t*-test).

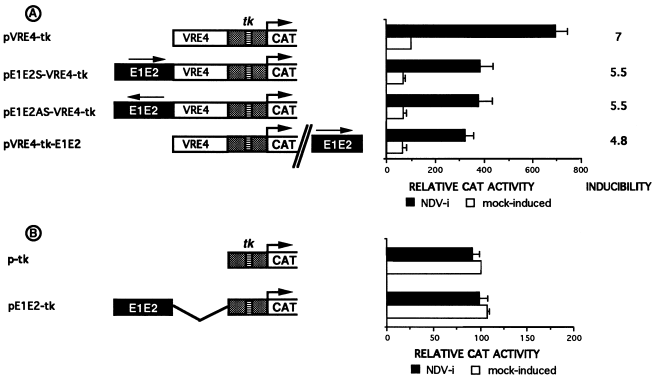

Figure 5 The effect of E1E2 is orientation- and position-independent but requires the VRE4

The names and structures of the different chimaeric plasmids tested are presented in the left-hand panel. Assay conditions are as described in the legend to Figure 1. (**A**) The effect of E1E2 is orientation- and position-independent. The arrows on the E1E2 box represent the orientation of the oligonuceotide in pVRE4. Histogram bars in the right-hand panel represent CAT activities ± S.E.M. for at least five separate transfections relative to the mock-induced activity of pVRE4 which was set at 100%. (**B**) The E1E2 sequence is ineffective in repressing the constitutive *tk* promoter activity. The p-*tk* plasmid corresponds to pBLCAT2 vector. Histogram bars represent CAT activities ± S.E.M. for four separate transfections relative to the mock-induced activity of p-*tk* which was set at 100%.

The presence of E1E2 is required for efficient repression of the native IFN-A11 promoter

The results described above show that the BC segment in a chimaeric construct has a repressing effect on the VRE4-mediated virus inducibility and that protein(s) bind to the BC segment at the E1E2 site. The requirement of the E1E2 sequence for efficient repression was determined by examination of the effect of this sequence in the native IFN-A11 promoter. In the experiments depicted in Figure 4, after virus induction, the E1E2-containing IFN-A11 promoter (−244/+19) shows the same level of transcriptional repression as the total (−457/+19) promoter. Deletion of the 46-bp E1E2 fragment in plasmid p(−199/+19)-CAT shows a derepression of the virus-induced transcriptional level and inducibility. The fully responding −119/+19 IFN-A11 promoter construct shows the same level of induced transcription. However, for the same mock-induced level, the inducibility of the E1E2-deleted IFN-A11 promoter (17-fold) is not sufficient to recover a similar inducibility to that of the −119/+19 IFN-A4 promoter (more than 50-fold). From these results, we conclude that the repression of the Mu IFN-A11 gene is in part due to the negative effect of the E1E2 sequence.

The repressing activity of E1E2 is orientation- and position-independent and is dependent on the presence of the VRE

The functional features of the 46-bp E1E2 sequence on promoter activity were further investigated by addressing the question as to whether E1E2 could act as a silencer. The effect of E1E2 was first tested for its repressing activity and placed either in the sense or the antisense orientation, upstream of the VRE4 in the pVRE4 reference plasmid. The results of transient-transfection experiments, as presented in Figure 5(A), show that E1E2 displays a repressing activity on the VRE4-mediated virus-induced activation of the heterologous promoter. The E1E2 sequence is also able to repress the viral-induced transcription to the same extent when placed in the opposite orientation.

To check if the effect of E1E2 could be exerted in a position-independent manner, the E1E2 sequence was placed downstream of the CAT gene. The results in Figure 5(A) show that the repression exerted by the E1E2 sequence is position- and orientation-independent.

Finally, the ability of the E1E2 sequence to act as a general silencer or in conjunction with other positively regulating elements was tested. The E1E2 oligonucleotide was inserted upstream of the constitutive heterologous *tk* promoter and assayed with or without virus induction. As shown in Figure 5(B), insertion of the E1E2 element had no effect on CAT gene expression under the control of the *tk* promoter.

The absence of repression of E1E2 on the *tk* promoter raises the question as to whether the negative effect of E1E2 is achieved through any domain that confers virus-inducibility or more particularly the VRE-A. To address this issue the VRE4 (−109/−64) was replaced by the human IFN-B IRE sequence (−80/−37) containing the elements necessary for response to virus induction [11]. The IFN-B IRE and the IFN-A VRE, although presenting some sequence homologies, are not constituted by the same arrangement of elements. They differ

Table 1 The E2 sequence displays the same repressing activity as E1E2

Assay conditions are as described in the legend to Figure 1. Inducibility is the ratio of the NDV-induced activity over the mock-induced activity.

Plasmids	NDV-induced*	Mock-induced*	Inducibility
pVRE4	700 ± 69	100	7
pE1E2-VRE4	385 ± 60	70 ± 12	5.5
pE2-VRE4	371 ± 55	70 ± 10	5.3

* Numbers represent CAT activities ± S.E.M. for four separate transfections relative to the mock-induced activity of pVRE4 which was set at 100%.

especially by the involvement or not of at least one *trans*-acting factor in the viral-induced response which is NF-κB. The E1E2 sequence had no effect on the virus-induced activity and inducibility of the IRE-mediated response to virus stimulation (results not shown). From these experiments we conclude that the presence of an IFN-A VRE seems to be required for functional repressing activity via E1E2.

The E1E2 sequence contains an IRF-binding site recognized by IRF2

E1E2 contains overlapping sequences which show homology with several regulatory elements acting negatively on gene transcription. Among them, E1E2 shows homology with the mouse N-*ras* NRE [53], the putative binding site for YY1 [57], and the consensus IRF-binding site [58]. The mouse N-*ras* NRE, in contrast to the E1E2 sequence, represses the *tk* promoter activity in L cells, and competition with an oligonucleotide corresponding to the YY1 binding site in gel shift assays failed to displace any shifted complex formed on the E1E2 sequence (results not shown). In the E2 subregion, the consensus IRF-binding site (Figure 3B) presents a nucleotide substitution [**G**(A)AAAG/CT/CGAAAG/CT/C]. This sequence is also homologous to the PRDI and PRDIII elements described in the Hu IFN-B promoter (GAGAAGTGAAAGTG and GAAAACTGAAAGG respectively) and to the PRDI-like sequence (GAAAGTGAAAAG) present in the VRE11 and VRE4. The PRDI sequence is the binding site for known *trans*-regulators such as IRF1, IRF2, PRDI-BF1 and ISGF3γ [8,23,59] which, it has been proposed, are involved in the transcriptional regulation of the Hu IFN-B gene. Recent reports seem to indicate that IRF2 can display a repressing effect alone without any antagonistic effect on IRF1 [60]. In light of these observations, the participation of IRF2 in the repression mediated by E1E2 is worth consideration because this protein is known to be present in uninduced L929 cells and its expression increases after virus induction [23,61,62]. Since the repressing effect of E1E2 is more marked in the induced conditions, IRF2 may be a potential candidate for this effect.

The functional effect of the E2 subregion was first tested on a VRE4-containing virus-inducible promoter. As shown in Table 1, the E2 subregion alone displays its negative effect on the activity of the chimaeric promoter as well as the whole E1E2 sequence. The repressing activity of the E2 oligonucleotide may be due to, at least in part, the binding of a repressing IRF protein member to the consensus element.

The ability of IRF2 to bind the E2 sequence was further investigated. As shown in Figure 6 (left-hand panel), in gel shift assays the recombinant Hu IRF2 (recHuIRF2) protein binds the E2 oligonucleotide probe efficiently and, in the presence of

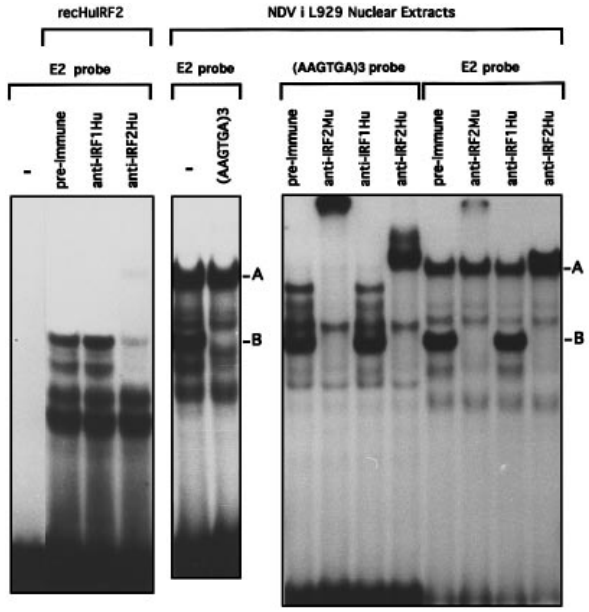

Figure 6 IRF2 binds the E2 sequence

Gel retardation assays were performed with 50000 c.p.m. per lane of either the E2 or $(AAGTGA)_3$ ^{32}P-labelled oligonucleotide probes. Binding, supershift and migrating conditions were as decribed in the Materials and methods section. Left-hand panel: the E2 probe was incubated with 1 ng of recHuIRF2 in the absence or presence of 1:10 dilution of anti-(Hu IRF1) or anti-(Hu IRF2) antibodies. The two faster-migrating bands correspond to proteolysis forms of the recHuIRF2 protein. Central panel: the E2 probe was incubated with 5 μg of NDV-induced L929 nuclear extracts. The B complex was efficiently competed by a 250-fold molar excess of $(AAGTGA)_3$ oligonucleotide. Right-hand panel: the $(AAGTGA)_3$ or E2 probes were incubated with 5 μg of NDV-induced L929 nuclear extracts in the presence of either anti-(Mu IRF2) (1:1 dilution) or anti-(Hu IRF1) or anti-(Hu IRF2) antibodies (1:10 dilution).

anti-(Hu IRF2) antibodies, the complex formation between the recombinant protein and the E2 probe was supershifted. Anti-(Hu IRF1) antibodies were unable to cross-react with the recHuIRF2. The recHuIRF2 also binds to the E1E2 sequence (results not shown). These results indicate that the IRF-binding site found in the E2 subregion is actually a binding site for the recHuIRF2 protein. Gel-retardation assays with the E2 probe show the same migration pattern consisting of two major complexes A and B formed either with virus-induced (Figure 6, central panel) or non-induced L929 cell nuclear extracts. When the $(AAGTGA)_3$ oligonucleotide, corresponding to the high-affinity IRF-binding site, was used as competitor, a major complex B was efficiently displaced. This complex, as shown in Figure 6 (right-hand panel), is supershifted in the presence of either anti-(Hu IRF2) or anti-(Mu IRF2) antibodies, suggesting the presence in the B complex of IRF2 or a protein of similar antigenicity. As a control, the $(AAGTGA)_3$ oligonucleotide was used as a probe in the presence of L929 NDV-induced nuclear extracts (Figure 6, right-hand panel). The complex corresponding to IRF2 co-migrates with the complex B.

We have further determined the molecular size of E2-binding proteins in the A and B complexes. The A complex is not displaced by competition with $(AAGTGA)_3$, is not supershifted by anti-IRF2 antibodies and, thus, does not seem to contain IRF2. UV cross-linking of the E2 probe to virus-induced L929 cell proteins reveals a single protein component of approx. 110 kDa (Figure 7). For the B complex, the UV cross-linking under the same conditions identifies a single protein of approx. 50 kDa, similar in molecular size to the IRF2 protein [61,63]. We

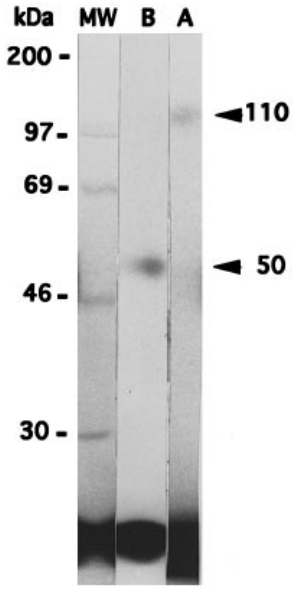

Figure 7 Identification of the E2-binding proteins by UV cross-linking

SDS/PAGE analysis of the E2-specific complexes A and B. A preparative gel-retardation assay was performed with NDV-induced L929 cell nuclear extracts and an internally labelled 5-bromodeoxyuridine-substituted E2C probe. The protein–DNA A and B complexes were UV cross-linked in the gel, excised, eluted and resolved by SDS/10%-PAGE. The excised A and B complexes and the molecular-mass markers (MW) are specified above each lane, and the labelled cross-linked proteins are denoted by arrowheads. The molecular masses of the markers and the cross-linked proteins (in kDa) are indicated.

can thus conclude that, in L929 cells, IRF2 is able to bind the IRF-binding site found in the E2 sequence and that another IRF-unrelated 110 kDa protein also binds this sequence.

DISCUSSION

The Mu IFN-A11 gene is weakly induced by NDV in L929 cells. In our previous studies with this cell line, we have demonstrated the presence of negatively acting sequences in the upstream regions of the Mu IFN-A11 gene promoter. In the present study, we have delimited an E1E2 ($-244/-199$) sequence which is able to reduce, in either orientation or position, both the transcriptional activity and inducibility of a virus-responsive promoter. However, this sequence is not able to reduce the transcriptional activity of a VRE-lacking promoter. We have shown that the binding of specific and rate-limiting proteins to E1E2 is responsible for the negative effect.

The Mu IFN-A11 gene is the first example in the Mu IFN-A family in which a distal negative promoter element has been described. The presence of negative regions, upstream of the Hu IFN-A1 VRE, acting constitutively has been suggested previously [31,32]; however, these domains have not been studied further. In any case, these regions were unable to prevent the response to viral induction. Here we show that the E1E2 sequence is able to repress mainly the induced expression of a VRE-A-mediated promoter. The Mu IFN-A6 gene is another member of the multigenic family and does not respond to virus induction in L cells [43]. It has been reported that its uninducibility is not due to the presence of distal negative elements but rather to an inefficient inducible element IE [37,38].

In an attempt to investigate the E1E2-binding factors, we first searched for homologies within the E1E2 sequence for known negative elements which are binding sites for repressor proteins. We found a homology to the consensus sequence for the IRF family of transcription factors which all share homologous DNA-binding domains. To date eight genes, members of this family, have been cloned and characterized [23,64–69]. While six members have been described as transcriptional activators, two of them display negative effects. ICSBP is a repressor which inhibits the expression of IFN-stimulated genes; its expression pattern is restricted to cells of the lymphoid and macrophage lineages [64]. The other member, the IRF2 protein, is known for its repressing effect due mostly to competition with IRF1 for binding to the same site [23]. It has also been reported that the IRF2 protein contains a repression domain in its C-terminal portion which is able to inhibit nearby activators or general transcription factors [60]. More recently, in the human histone H4 gene promoter, a transcriptional activating role has been ascribed to IRF2 [63]. Therefore, it seems that IRF2 displays dual activator/repressor functions depending on the promoter context. We have shown that recombinant IRF2 binds to an element in E1E2. Supershift assays with anti-IRF2 antibodies and UV cross-linking experiments demonstrated that in NDV-induced L929 cell nuclear extracts, the IRF2 molecules bind to the IRF-binding site in E1E2. Since E2 and E1E2 in chimeric constructs display their negative effects to the same extent (Table 1), it can be deduced that the major elements necessary for repression are present in the E2 sequence. One of these elements is the IRF-binding site, although it is not clear that IRF2 is acting on its own. The other, still unidentified, element is the binding site for the 110 kDa protein. This protein may be a potential repressor candidate or IRF2-partner for the E2-mediated repression (Figure 6). The participation of IRF2 with the 110 kDa protein in a multicomponent repressing complex and their actual involvement in the modulation of Mu IFN-A11 promoter repression remain to be established.

The repressing features of the E1E2 sequence are on the one hand close to the definition of a silencer in that the negative effect is observed in either orientation and is position independent. On the other hand, the E1E2 sequence is unable to repress a constitutive *tk* promoter by direct interaction with the activators (e.g. Sp1) or with the basal transcriptional machinery. We can thus rule out a mechanism in which repression is exerted on the general transcriptional machinery. Therefore, the E1E2 negative sequence cannot be considered strictly a general silencer because it specifically acts by repressing the VRE-mediated induced activity. This specificity is not due to the requirement for any virus-inducible element. Indeed, the E1E2 sequence is ineffective on the IFN-B IRE-mediated virus induction. These results indicate that to exert its effect, E1E2 seems to require specific interactions with virus-activated positive factors, thus affecting the activating mechanisms displayed by the VRE-A. Evidence which further supports this notion includes the facts that: (i) the E1E2 sequence has no effect on a VRE-lacking promoter. Nevertheless, the same E1E2 footprint patterns are observed whether or not the probes used contain the VRE. These observations suggest that the absence of the effect of E1E2 on *tk* promoter is not due to the lack of repressor binding but rather the negative factors bound to E1E2 may act either directly or indirectly on the VRE-mediated activation. (ii) In the transient-transfection experiments, the repression in the induced conditions appears more marked. Under these conditions, virus-activated *trans*-activators bind to the VRE-A. Therefore, a relation between these two regions may be that the factors which bind to one and/or the other region may interact more strongly upon induction.

All together, these observations raise the possibility that several

mechanisms may account for this repression. A possible mechanism could be a quenching-type repression. Indeed the negative *trans*-regulator(s) on E1E2 may interact with VRE-binding activator(s) and inhibit its positive effect. Such a mechanism has recently been proposed for the cyclic AMP-inducible promoter in the rat lactate dehydrogenase A subunit gene [70]. In this promoter, as in the IFN-A11 promoter, a distal NRE displays its effect independently of distance or orientation, and yet has an absolute requirement for the presence of a cAMP-responsive element module. It has been suggested that the factors binding to the NRE may interact with those binding to the cAMP-responsive element for efficient repression. Other possible mechanisms could also contribute to the E1E2-mediated repression; for instance, it may involve steric occlusion, not by competition for the same binding site as classically described between an activator and a repressor, but rather by a 'direct effect' exerted by the same protein containing two DNA-binding domains [13,71]. One of the domains may bind to E1E2 and the other to an element in the VRE and thus prevent the binding of positive virus-activated factors to the latter element.

In order to gain insight into the repressing mechanisms which control the E1E2-mediated negative effect, we are currently investigating the different partners of this interaction. On the one hand, the understanding of the mechanisms underlying viral induction via the VRE and, on the other hand, the identification of the E1E2 binding factors are prerequisite conditions. In this respect, it will be of interest to further investigate the 110 kDa protein in the A complex and then, in a second step, to examine its potential interaction with IRF2.

We are grateful to Dr. Luckow for providing us with pBLCAT2, to Dr. D. Biard and Dr. J. Mushler for the pCMV-βGal and pEF-LacZ plasmids respectively, and to Dr. T. Taniguchi and Dr. N. Watanabe for generously providing us with the murine anti-IRF2 antibodies. We are indebted to Dr. J. Hiscott for his gift of the recHuIRF2 protein and the anti-(Hu IRF1 and IRF2) antibodies but also for valuable discussions and comments in preparing the manuscript. We thank Dr. S. Cereghini for helpful discussions and Dr. S. Pellegrini for critical reading of the manuscript. Special thanks to M. Grandin for her skilful technical assistance. This work was supported by the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer (Contrat No. 1042) and the Ligue Nationale Française contre le Cancer.

REFERENCES

1 Renkawitz, R. (1990) Trends Genet. **6**, 192–196
2 Goodbourn, S. (1990) Biochim. Biophys. Acta **1032**, 53–77
3 Clark, A. R. and Docherty, K. (1993) Biochem. J. **296**, 521–541
4 Herschbach, B. M. and Johnson, A. D. (1993) Annu. Rev. Cell Biol. **9**, 479–509
5 Weidle, U. and Weissmann, C. (1983) Nature (London) **303**, 442–446
6 Raj, N. B. K. and Pitha, P. M. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3923–3927
7 Raj, N. B. K. and Pitha, P. M. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 7426–7430
8 Keller, A. D. and Maniatis, T. (1991) Genes Dev. **5**, 868–879
9 Whittemore, L.-M. and Maniatis, T. (1990) Mol. Cell. Biol. **10**, 1329–1337
10 Goodbourn, S., Zinn, K. and Maniatis, T. (1985) Cell **41**, 509–520
11 Goodbourn, S., Burnstein, H. and Maniatis, T. (1986) Cell **45**, 601–610
12 Fan, C.-M. and Maniatis, T. (1989) EMBO J. **8**, 101–110
13 Maniatis, T., Whittemore, L.-M., Du, W., Fan, C.-M., Keller, A. D., Palombella, V. J. and Thanos, D. (1992) in Transcriptional Regulation (McKnight, S. and Yamamoto, K., eds.), pp. 1193–1220, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
14 Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y. and Taniguchi, T. (1988) EMBO J. **7**, 3397–3405
15 Visvanathan, K. V. and Goodbourn, S. (1989) EMBO J. **8**, 1129–1138
16 Hiscott, J., Alper, D., Cohen, L., Leblanc, J. F., Sportza, L., Wong, A. and Xanthoudakis, S. (1989) J. Virol. **63**, 2557–2566
17 Lenardo, M. J., Fan, C. M., Maniatis, T. and Baltimore, D. (1989) Cell **57**, 287–294
18 Fujita, T., Miyamoto, M., Kimura, Y., Hammer, J. and Taniguchi, T. (1989) Nucleic Acids Res. **17**, 3335–3346
19 Thanos, D. and Maniatis, T. (1992) Cell **71**, 777–789
20 Du, W. and Maniatis, T. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 2150–2154
21 Du, W., Thanos, D. and Maniatis, T. (1993) Cell **74**, 887–898
22 Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T. and Taniguchi, T. (1988) Cell **54**, 903–913
23 Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T. and Taniguchi, T. (1989) Cell **58**, 729–739
24 Leblanc, J.-F., Cohen, L., Rodrigues, M. and Hiscott, J. (1990) Mol. Cell. Biol. **10**, 3987–3993
25 Matsuyama, T., Kimura, T., Kitagawa, M., Pfeffer, K., Kawakami, T., Watanabe, N., Künding, T. M., Amakawa, R., Kishihara, K., Wakeham, A., Potter, J., Furlonger, C. L., Narendran, A., Suzuki, H., Ohashi, P. S., Paige, C. J., Taniguchi, T. and Mak, T. W. (1993) Cell **75**, 83–97
26 Ruffner, H., Reis, L. F. L., Näf, D. and Weissmann, C. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 11503–11507
27 Kimura, T., Nakayama, K., Penninger, J., Kitagawa, M., Harada, H., Matsuyama, T., Tanaka, N., Kamijo, R., Vilcek, J., Mak, T. W. and Taniguchi, T. (1994) Science **264**, 1921–1924
28 Reis, L. F. L., Ruffner, H., Stark, G., Aguet, M. and Weissmann, C. (1994) EMBO J. **13**, 4798–4806
29 Nourbakhsh, M., Hoffmann, K. and Hauser, H. (1993) EMBO J. **12**, 451–459
30 Lehming, N., Thanos, D., Brickman, J. M., Ma, J., Maniatis, T. and Ptashne, M. (1994) Nature (London) **371**, 175–179
31 Kuhl, D., de la Fuente, J., Chaturvedi, M., Parimoo, S., Ryals, J., Meyer, F. and Weissmann, C. (1987) Cell **50**, 1057–1069
32 Ryals, J., Dierks, P., Ragg, H. and Weissmann, C. (1985) Cell **41**, 497–507
33 Raj, N. B. K., Israeli, R., Kellum, M. and Pitha, P. M. (1989) J. Biol. Chem. **264**, 11149–11157
34 MacDonald, N. J., Kuhl, D., Maguire, D., Näf, D., Gallant, P., Goswamy, A., Hug, H., Büeler, H., Chaturvedi, M., de la Fuente, J., Ruffner, H., Meyer, F. and Weissmann, C. (1990) Cell **60**, 767–779
35 Hiscott, J., Cantell, K. and Weissmann, C. (1984) Nucleic Acids Res. **12**, 3727–3746
36 Asano, M., Hayashi, M., Yoshida, E., Kawade, Y. and Iwakura, Y. (1990) Virology **176**, 30–38
37 Raj, N. B. K., Au, W. C. and Pitha, P. M. (1991) J. Biol. Chem. **266**, 11360–11365
38 Au, W. C., Raj, N. B. K., Pine, R. and Pitha, P. M. (1992) Nucleic Acids Res. **20**, 2877–2884
39 Au, W. C., Su, Y., Raj, N. B. K. and Pitha, P. M. (1993) J. Biol. Chem. **268**, 24032–24040
40 Genin, P., Bragança, J., Darracq, N., Doly, J. and Civas, A. (1995) Nucleic Acids Res. **23**, 5055–5063
41 Zwarthoff, E. C., Moren, A. T. A. and Trapman, J. (1985) Nucleic Acids Res. **13**, 791–804
42 Kelley, K. A. and Pitha, P. M. (1985) Nucleic Acids Res. **13**, 825–839
43 Bisat, F., Raj, N. B. K. and Pitha, P. M. (1988) Nucleic Acids Res. **16**, 6067–6083
44 Hoss-Homfeld, A., Zwarthoff, E. C. and Zawatzky, R. (1989) Virology **173**, 539–550
45 Dion, M., Vodjdani, G. and Doly, J. (1986) Biochem. Biophys. Res. Commun. **138**, 826–834
46 Navarro, S., Dion, M., Vodjdani, G., Berlot-Picard, F. and Doly, J. (1989) J. Gen. Virol. **70**, 1831–1839
47 Coulombel, C., Vodjdani, G. and Doly, J. (1991) Gene **104**, 187–195
48 Civas, A., Dion, M., Vodjdani, G. and Doly, J. (1991) Nucleic Acids Res. **19**, 4497–4502
49 Luckow, B. and Schütz, G. (1987) Nucleic Acids Res. **15**, 5490
50 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
51 Wigler, M., Pellicer, A., Siverstein, S. and Axel, R. (1978) Cell **14**, 725–731
52 Nordeen, S. K., Green III, P. P. and Fowkles, D. M. (1987) DNA **6**, 173–178
53 Paciucci, R. and Pellicer, A. (1991) Mol. Cell. Biol. **11**, 1334–1343
54 Dignam, J. D., Lebowitz, R. M. and Roeder, R. G. (1983) Nucleic Acids Res. **11**, 1475–1489
55 Lin, R., Mustafa, A., Nguyen, H., Gewert, D. and Hiscott, J. (1994) J. Biol. Chem. **269**, 17542–17549
56 Cereghini, S., Blumenfeld, M. and Yaniv, M. (1988) Genes Dev. **2**, 957–974
57 Hariharan, N., Kelley, D. E. and Perry, R. P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 9799–9803
58 Tanaka, N., Kawakami, T. and Taniguchi, T. (1993) Mol. Cell. Biol. **13**, 4531–4538
59 Kawakami, T., Matsumoto, M., Sato, M., Harada, H., Taniguchi, T. and Kitagawa, M. (1995) FEBS Lett. **358**, 225–229
60 Yamamoto, H., Lamphier, M. S., Fujita, T., Taniguchi, T. and Harada, H. (1994) Oncogene **9**, 1423–1428
61 Palombella, V. J. and Maniatis, T. (1992) Mol. Cell. Biol. **12**, 3325–3336
62 Watanabe, N., Sakakibara, J., Hovanessian, A. G., Taniguchi, T. and Fujita, T. (1991) Nucleic Acids Res. **19**, 4421–4428
63 Vaughan, P. S., Aziz, F., van Wijnen, A. J., Wu, S., Harada, H., Taniguchi, T., Soprano, K. J., Stein, J. L. and Stein, G. S. (1995) Nature (London) **377**, 362–365

64 Driggers, P. H., Ennist, D. L., Gleason, S. L., Mak, W.-H., Marks, M. S., Levi, B.-Z., Flanagan, J. R., Appella, E. and Ozato, K. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3743–3747
65 Veals, S. A., Schindler, C., Leonard, D., Fu, X.-Y., Aebersold, R., Darnell, Jr., J. E. and Levy, D. E. (1992) Mol. Cell. Biol. **12**, 3315–3324
66 Matsuyama, T., Grossman, A., Mittrücker, H.-W., Siderovski, D. P., Kiefer, F., Kawakami, T., Richardson, C. D., Taniguchi, T., Yoshinaga, S. K. and Mak, T. W. (1995) Nucleic Acids Res. **23**, 2127–2136

Received 7 February 1996/18 March 1996; accepted 19 March 1996

67 Grant, C. E., Vasa, M. Z. and Deeley, R. G. (1995) Nucleic Acids Res. **23**, 2137–2146
68 Au, W.-C., Moore, P. A., Lowther, W., Juang, Y.-T. and Pitha, P. M. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 11657–11661
69 Eisenbeis, C. F., Singh, H. and Storb, U. (1995) Genes Dev. **9**, 1377–1387
70 Chung, K. C., Huang, D., Chen, Y., Short, S., Short, M. L., Zang, Z. and Jungmann, R. A. (1995) Mol. Cell. Biol. **15**, 6139–6149
71 Fan, C.-M. and Maniatis, T. (1990) Genes Dev. **4**, 29–42