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An ATF/CREB binding site protein is required for virus induction of the human interferon β gene

(interferon β gene regulation/cAMP)

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ABSTRACT **We report the characterization of a distinct regulatory element of the human interferon β (HuIFN-β) gene promoter, which we designate PRDIV (positive regulatory domain IV). In previous studies, sequences between −104 and −91 base pairs upstream from the start site of transcription were shown to be required for maximal levels of virus induction in mouse L929 cells. We have localized the essential sequence in this region extending from −99 to at least −91, and we show that this sequence is a binding site for a protein of the activating transcription factor/cAMP response element binding protein (ATF/CREB) family of transcription factors. Mutations in PRDIV that decrease the affinity of one member of this family (ATF-2/CRE-BP1) decrease the level of virus induction *in vivo*. Moreover, multiple copies of PRDIV can confer both virus and cAMP inducibility upon a minimal promoter in L929 cells, while it is constitutively active in HeLa cells. We conclude that PRDIV is a distinct regulatory element of the HuIFN-β promoter and that the signal transduction pathways involved in virus and cAMP induction may partially overlap.**

Expression of the human interferon β (HuIFN-β) gene is highly inducible by both virus and double-stranded RNA (1). Studies of the sequence requirements for induction revealed cell type-specific differences (2). Specifically, only 77 base pairs (bp) of promoter sequences upstream from the transcription start site is required for virus induction in mouse C127 cells, while 104 bp is required in mouse L929 cells (3–6). As shown in Fig. 1, two distinct virus-inducible elements were identified in the −77 promoter. The positive regulatory domain I (PRDI) is located between nucleotides −77 and −64 from the start site of transcription, while another element (PRDII) is located between −66 and −55 (7–10). A third element (PRDIII) is located between −90 and −78 (11–15).

The −104 and −91 region of the HuIFN-β promoter, which we designated PRDIV, is essential for high levels of virus induction in mouse L929 cells (5, 6). Comparison of the DNA sequence in this region with known protein binding sites reveals three potential regulatory sequences (see Fig. 1). First, a potential IRF-1-like binding site is located between −103 and −91 (16, 17). Second, an Oct-1 site (18) is located between −105 and −98. This site was previously shown to specifically bind to the Oct-1 protein *in vitro* (A. Keller and T.M., unpublished data). Third, an ATF-CREB site is located between −99 and −91. This ATF/CREB site fits with the consensus 5′-GTGACGTA/CA/G-3′ except at position −94 (underlined), which has an A instead of a G (19, 20). We find that recombinant ATF/2CRE-BP1 protein produced in bacteria (21, 22) binds specifically to this site (unpublished data).

To determine which of the three potential regulatory elements in PRDIV is required for virus induction in L929 and HeLa cells, we introduced point mutations into this region and then tested their effect on virus induction in transient transfection assays. We found that the ATF/CREB site, but not the IRF-1-like site or the Oct motif, is required for virus induction in both L929 and HeLa cells. In addition, we found that multiple copies of PRDIV are able to confer both virus and cAMP induction in L929 cells but are constitutively active in HeLa cells. Thus, PRDIV is a distinct regulatory element required for maximal levels of virus induction of the HuIFN-β gene. These observations suggest that the regulatory pathways involved in virus and cAMP induction may partially overlap.

MATERIALS AND METHODS

Oligonucleotides. MT-a, 5′-GATCTCTCTCTATTCAGAGGAATTTCCCACTTTCACTTCTCCCTTTCAGTTTTCCTATGTCXYYYXZXYTTTAGG-3′ (75-mer); MT-s, 5′-GATCCCTAAAXYQYXXXYGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCTGAATAGAGAGA-3′ (75-mer) (X is 90% A + 3.33% each G + C + T, Y is 90% T + 3.33% each G + C + A, Z is 90% C + 3.33% each A + T + G, and Q is 90% G + 3.33% each A + T + C); MTCRE, 5′-CGGGATCCTAAAATGTAAAT(G,T)(A,C)C(A,G)TAGGAA-3′ (30-mer); RES, 5′-GACTCTAGAGGATCTGAATTCCATGACATAGGAAAACTGAAA-3′ (42-mer); MTPRDIII, 5′-AGAGGATCCTAAAATGTAAATGACATAG(G,T)(A,C)(A,G)(A,C)CTG-3′ (36-mer); PRDIV-s, 5′-GATCCTGTAAATGACATAGGAAAA-3′ (24-mer); PRDIV-a, 5′-GATCTTTTCCTATGTCATTTACAG-3′ (24-mer); CRE, 5′-AATTCTGACGTCAG-3′ (14-mer).

Mutagenesis. Mutants in the Oct motif were made by clone annealed MT-a/MT-s oligonucleotides into a −40 IFN chloramphenicol acetyltransferase (CAT) construct linearized with *Bam*HI. Mutants in the ATF/CREB site and PRDIII were made by PCR, using *Hin*dIII linearized WT-1 from Fig. 2 as template and SP6 and MTCRE or MTPRDIII as primer. PCR products were digested with *Bam*HI and *Cla* I, and cloned into −40 HuIFN-β CAT digested with *Bam*HI and *Cla* I. The −99 IFN CAT constructs were made as described above, except the *Hin*dIII linearized −91 IFN CAT was used as a template, and SP6 and RES were used as primers. Cloning procedures were as described (23).

Cell Culture, DNA Transfection, CAT Assay, and RNase Mapping. Cells were maintained as described (7). Transfections were done by either the DEAE-dextran (24) or the calcium phosphate (25) method. The calcium phosphate method is used unless otherwise indicated. Cells were induced with virus (3) or 1 mM 8-bromo-cAMP, or they were mock induced. CAT assay and RNase mapping were done as described (7). All the data shown have been repeated at least three times.

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Abbreviations: HuIFN-β, human interferon β; PRD, positive regulatory domain; IRF-1, interferon regulatory factor 1; ATF/CREBP, activating transcription factor/cAMP response element binding protein; CAT, chloramphenicol acetyltransferase; CHX, cycloheximide.

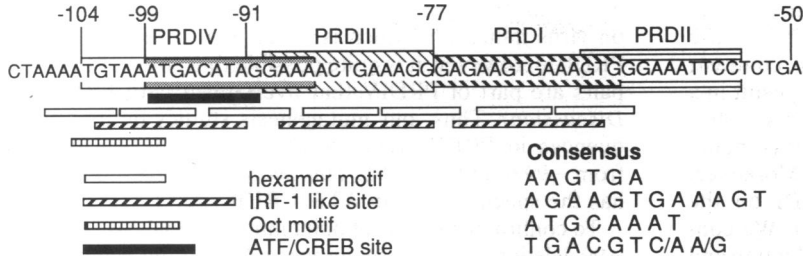

FIG. 1. Organization of the HuIFN-β gene promoter. Nucleotide sequence between -50 and -110 bp upstream from the transcription start site is shown. The four boxes indicate regulatory sequences of the HuIFN-β promoter designated PRDI, PRDII, PRDIII, and PRDIV (2). The shaded part of PRDIV represents critical sequences that affect virus induction in mutagenesis analysis.

DNA Binding Assay. Mouse ATF-2/CRE-BP1 was isolated from an L929 cDNA expression library, and recombinant protein was made with the T7 expression system (26). A gel retardation assay was performed as described (22). Binding of Oct-1 to the mutant probes was done by incubating 6 μg of HeLa nuclear extract with different mutant probes in the presence of 50 ng each of PRDI, PRDII, and PRDIV unlabeled oligonucleotides. The shifted bands were identified as Oct-1-DNA complexes by anti-Oct-1 antibody, by comparing the mobility with purified Oct-1, and by competition with an octamer oligonucleotide.

RESULTS

The ATF/CREB Site of the HuIFN-β Promoter Is Required for Virus Induction in Mouse L929 Cells. To determine which of the regulatory sequence motifs in PRDIV are required for virus induction (Fig. 1), we introduced point mutations into this region and analyzed their effects on virus induction in the context of the -110 IFN promoter and on their affinity for Oct-1 protein and ATF-2/CREBP-1 *in vitro*. The results of these studies are presented in Fig. 2 and are summarized below.

Mutations in the octamer motif. A number of mutations that decreased the affinity of the HuIFN-β promoter for Oct-1 protein *in vitro* had no effect on virus induction in L929 cells. For example, the octamer motif is altered in mutants -105C, -104C, -103A, -102G, -101C, and -100G. All of these mutations significantly decreased the affinity of Oct-1 protein for this sequence *in vitro* (Fig. 2*B*), but none of them decreased the level of virus induction in L929 cells (Fig. 2*A*). The -102C mutation creates a perfect Oct site in the HuIFN-β promoter, and it resulted in an increase in the affinity of the Oct-1 protein *in vitro*. However, virus induction was not affected. The mutation -102G decreased the affinity of Oct-1 but actually resulted in a 3-fold increase in expression after virus induction. The base change in this mutant generates the sequence AAAATGGAAATG, which is virtually identical to the "TG" regulatory sequence GAAATGGAAATG (27). Whether the creation of this sequence is responsible for the observed increase in expression remains to be determined. Mutations -99T, -103A/-98G, and -105G/-93Δ decrease the affinity of the promoter for Oct-1, and they decrease the level of virus induction (Fig. 2*A*). However, the effect on virus induction is likely due to disruption of the overlapping ATF/CREB site (see below). We conclude that the binding site for Oct-1 is not required in virus induction in L929 cells.

Mutations in the IRF-1-like site. Previous studies suggest that IRF-1 may bind to the -104/-91 region (14, 16, 17). However, we cannot detect binding of IRF-1 to a PRDIV oligonucleotide (data not shown). Moreover, *in vitro* DNase I footprinting experiments with the -110 HuIFN-β promoter revealed protection of both PRDI and PRDIII, extending to -94 with a hypersensitive site at -96 (unpublished data); protection of the whole IRF-1-like site of the -104/-91 region was not observed.

Further evidence that the hexamer/IRF-1-like site in PRDIV is not involved in virus induction was provided by transfection studies. Mutants -105C, -104C, -103A, -101C, -100G, and -97T all contain base substitutions in the hexamer/IRF-1-like site in the -104/-91 region, but no

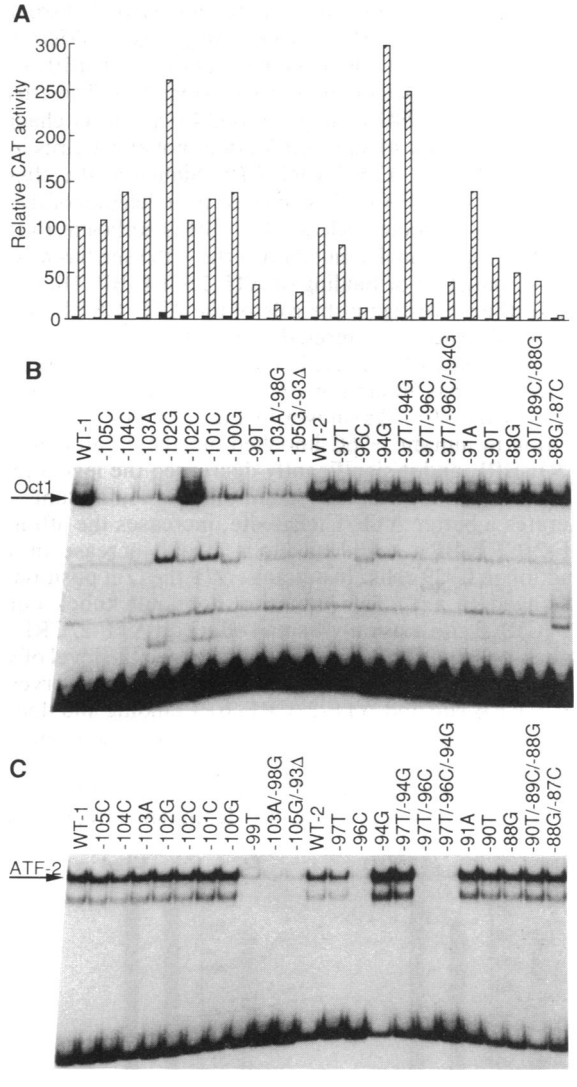

FIG. 2. Effects of point mutations in the HuIFN-β promoter on virus induction and binding to Oct-1 and ATF-2/CRE-BP1. (*A*) Histogram showing the level of CAT expression observed from L929 cells transfected with the indicated mutants. Solid and hatched bars indicate the level of CAT activity before and after virus induction, respectively. Each mutant is designated by a number corresponding to its location in the promoter and a letter indicating the nucleotide at this position. Δ indicates a deletion. Refer to Fig. 1 for wild-type (WT) sequences. WT-1 and WT-2 differ by 1 bp in the linker sequence. No differences in expression or binding were observed between the two constructs. Mutants in the Oct motif, including -99T, -103A/-98G, -105G/-93Δ, and -91A, are isogenic to WT-1; the rest are isogenic to WT-2. (*B*) Autoradiogram showing results of a gel shift experiment with wild-type and mutant DNA probes and Oct-1 protein. Arrow indicates the Oct-1 shifted bands. (*C*) Same as *B*, but with recombinant mouse ATF-2/CRE-BP1 protein.

effects on virus induction were observed. Interestingly, mutation −94G mutates the IRF-1-like site, but a 3-fold increase in the level of virus induction was observed. In contrast, mutations in the IRF-1 binding site of PRDIII did result in a decrease in virus induction. For example, mutants −90T, −88G, −90T/−89C/−88G are ≈2-fold less for virus induction, and mutant −88G/−87C is not inducible. Moreover, bacterially produced IRF-1 protected only PRDI on the −88G/−87C mutant promoter (unpublished data). We conclude that there is no correlation between virus induction and the presence of an IRF-1-like site in PRDIV, whereas such a correlation is observed in PRDIII.

Mutations in the ATF/CREB site. Mutations that disrupt the ATF/CREB site significantly decreased the level of virus induction in L929 cells. Indeed, mutations that disrupt the ATF/CREB site, −99T, −103A/−98G, and −105G/−93Δ, significantly decrease the binding of ATF-2/CRE-BP1 *in vitro* (Fig. 2*C*) and virus induction *in vivo* (Fig. 2*A*). The effect of mutant −103A/−98G is likely caused by a T to G change at position −98, since mutant −103A does not affect virus induction or binding of ATF-2/CRE-BP1. Similarly, the effect of mutant −105G/−93Δ is likely caused by a deletion of the T at −93, since a promoter deletion to −104 is sufficient for virus induction (Fig. 3), and a mutation at the −105 site has no effect on virus induction or binding of ATF-2/CRE-BP1 (Fig. 2).

To further investigate the role of the ATF/CREB element in virus induction, we altered the sequence to achieve a better or a worse match to the ATF/CREB consensus site (19, 20) and tested virus induction *in vivo* and binding of ATF-2/CRE-BP1 *in vitro*. As shown in Fig. 2 *A* and *C*, the point mutation in mutant −96C knocked out the binding of ATF-2/CRE-BP1, and it significantly decreased the level of virus induction in L929 cells. The point mutation in mutant −94G generates a better ATF/CREB site, increases the affinity of ATF-2/CRE-BP1, and leads to a 3-fold increase in virus induction in L929 cells. In mutant −97T the G at position −97 is changed to a T. This mutation does not knock out the ATF/CREB site, has only a small effect on ATF-2/CRE-BP1 binding, and does not significantly decrease the level of virus induction in L929 cells. The −91A mutation preserves the ATF/CREB site and ATF-2/CRE-BP1 binding and does not affect virus inducibility. Significantly, mutations between −105 and −100, which are located outside of the ATF/CREB-like element, do not affect the affinity of ATF-2/CRE-BP1 *in vitro*, and they have no significant effect on virus induction. Mutations between −90 and −87 do have an effect on virus induction, which is likely due to the disruption of PRDIII. However, it is quite possible that some of these base pairs are part of PRDIV that overlapped with PRDIII (see *Discussion*). Thus, our mutagenesis studies defined the sequences in PRDIV required for virus induction extended from −99 to at least −91. This sequence corresponds to and has the specificity of an ATF/CREB site.

To confirm the results of point mutation analysis, we made a −99 deletion construct by inserting ATGACATA immediately upstream of the −91 deletion. As shown in Fig. 3*A*, the −104 and −99 deletion constructs showed similar virus induction activity, the −91 deletion construct was ≈10-fold less active in response to virus induction, and no induction was observed for the −77 or −40 deletion constructs. Thus, inclusion of the ATF/CREB site completely restores the virus inducibility of the −91 deletion. This result is also consistent with a comparison of the human and mouse IFN-β promoter sequences (28), which shows that the Oct motif is not conserved, while the ATF/CREB site is virtually identical.

PRDIV Constitutes a Virus- and cAMP-Inducible Element in L929 Cells. To analyze the activity of PRDIV, we multimerized a PRDIV oligonucleotide and introduced from one to six copies upstream of the −77 HuIFN-β deletion construct. The resulting constructs were then tested for both virus and cAMP induction in L929 cells. As shown in Fig. 4*A*, the −77 promoter is not inducible by either virus or cAMP. Insertion of a single PRDIV element upstream of the −77 promoter leads to a small but detectable increase in the level of expression upon virus infection and a 2- to 3-fold increase in the level of expression upon cAMP induction. When additional copies of the oligonucleotide were inserted, a dramatic increase in both virus and cAMP induction was observed. The highest level of expression was observed with the plasmid containing six copies of PRDIV.

To compare the activity of PRDIV and that of the palindromic ATF/CREB site, we inserted six copies of such a CRE upstream from the −77 promoter and assayed for virus and cAMP inducibility. As shown in Fig. 5*A*, six copies of CRE confer both virus and cAMP inducibility on the −77 promoter as did six copies of PRDIV. Thus, in this promoter context, PRDIV and the CRE are functionally interchangeable. This observation also suggests that both elements are capable of interacting with PRDI and PRDII in the HuIFN-β promoter.

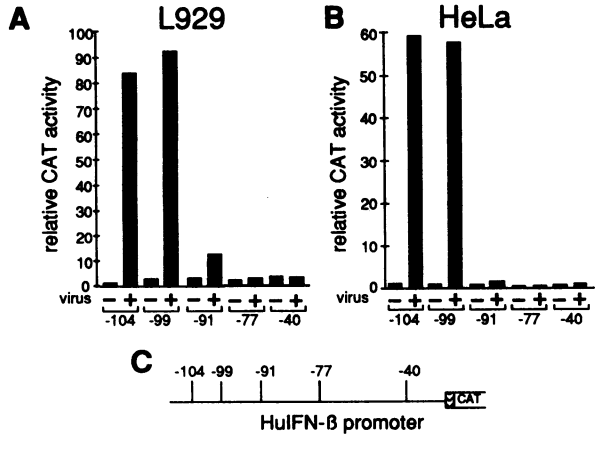

FIG. 3. Effects of HuIFN-β gene promoter deletions on virus induction in L929 and HeLa cells. (*A*) Histogram showing CAT activity produced in L929 cells after transfection with the indicated HuIFN-β deletion constructs. −, Mock induced; +, virus induced. −99 HuIFN-β deletion construct was made as described in *Materials and Methods*. (*B*) Same as *A*, except in HeLa cells. (*C*) Diagram of deletion constructs.

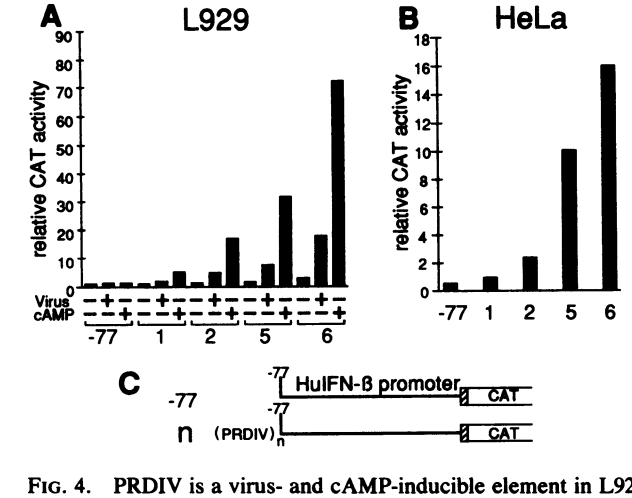

FIG. 4. PRDIV is a virus- and cAMP-inducible element in L929 cells and a constitutive element in HeLa cells. (*A*) Histogram showing the levels of CAT activity in L929 cells transfected with reporters diagramed in *C*. A single transfected plate was split into three and then either mock induced (−) or induced with virus (virus +) or cAMP (cAMP +). (*B*) Histogram showing the constitutive levels of CAT activity in HeLa cells. (*C*) Diagram of reporter constructs used. *n* indicates the construct with *n* copies of PRDIV.

To determine whether PRDIV can confer virus and cAMP inducibility on a heterologous promoter, we inserted six copies of PRDIV upstream of the TATA box of the adenovirus E1b promoter (29). As shown in Fig. 5*B*, the E1b TATA promoter is not inducible by either agent, but the construct containing six copies of PRDIV is highly inducible by cAMP and, to a lesser extent, by virus. Interestingly, multiple copies of the Oct motif, which partially overlaps with PRDIV, do not confer virus induction on a minimal TATA box promoter (data not shown). Significantly, the construct containing six copies of the CRE was highly inducible by cAMP, but not by virus (Fig. 5*B*). Similarly a construct with six copies of the ATF/CREB site of the HuIFN promoter (−99 to −91) is only weakly inducible by cAMP and is not virus inducible (data not shown). Thus, the ATF/CREB sites require flanking sequences for virus induction.

We performed RNase mapping studies to determine whether the increase in CAT activity on virus or cAMP induction results from an increase in the level of correctly initiated CAT mRNA. As shown in Fig. 5*C*, correctly initiated message is increased from the six PRDIV E1b constructs after virus or cAMP induction. When virus or cAMP induction was carried out in the presence of CHX, a higher level of CAT RNA was observed compared to induction in the absence of CHX (Fig. 5*D*). CHX alone is not sufficient to induce transcription from these constructs (Fig. 5*D*, lane 4). In contrast, no correctly initiated RNA was detected when the E1b TATA control plasmid was transfected into L929 cells (Fig. 5*D*). We conclude that virus or cAMP induction leads to an increase in the level of correctly initiated CAT mRNA and that protein synthesis is not required for either induction. In addition, the inhibition of protein synthesis appears to result in the stabilization of CAT message and/or increased transcription.

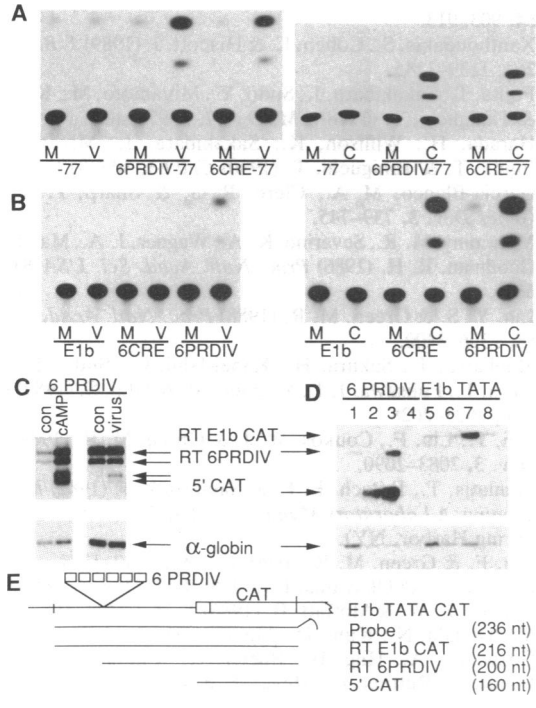

FIG. 5. Comparison of activities of PRDIV and the palindromic CRE. (*A*) Levels of CAT activity produced by L929 cells transfected with reporters containing six copies of PRDIV or CRE upstream from a −77 HuIFN-β promoter. Lanes: M, mock induced; V, virus induced; C, cAMP induced. Transfections were performed by the DEAE-dextran method for both *A* and *B*. More extracts were used when comparing virus induction to give optimal conversion. (*B*) Same as in *A* except that constructs with six copies of PRDIV or CRE upstream of the E1b TATA were used. (*C*) RNase mapping of RNA from L929 cells transfected with the six-PRDIV E1b CAT construct. (con) uninduced, cAMP induced for 2 hr, virus induced for 5 hr. Doublets of the protected bands are probably due to variations of RNase mapping. α-Globin was used to normalize transfection efficiency for both *C* and *D*. (*D*) RNase mapping of RNA from L929 cells transfected with the six-PRDIV E1b CAT or E1b CAT constructs. Lanes: 1 and 5, uninduced; 2 and 6, induced with virus and cycloheximide (CHX); 3 and 7, induced with cAMP and CHX; 4 and 8, induced with CHX alone. Induction time was 6 hr. (*E*) Diagram of the E1b TATA CAT probe. The full-length probe was 236 nucleotides (nt) long, the protected readthrough (RT) from E1b and six-PRDIV construct was 216 and 200 nt, respectively, and the correctly initiated message (5′ CAT) was 160 nt.

A Cell-Specific Difference in the Transcriptional Activity of PRDIV. The region between −104 and −91 is also required for induction in human HeLa cells (Fig. 3*B*). However, in contrast to L929 cells, the PRDIV element acts as a constitutive element in HeLa cells. As shown in Fig. 4*B*, increasing numbers of copies of PRDIV upstream of the HuIFN-β −77 promoter resulted in progressively higher levels of CAT activity in the absence of induction. Similarly, six copies of PRDIV led to high constitutive CAT activity on the E1b TATA construct (Fig. 6*B*). No further increase in the level of expression is observed for the six-PRDIV E1b construct with either virus or cAMP induction (Fig. 6*C*). The six-PRDIV −77 IFN construct is still slightly virus inducible in HeLa cells (Fig. 6*A*), and this is likely the result of interactions between PRDIV and the virus-inducible elements of PRDI and PRDII. Cell-specific activity of an ATF/CREB site has been reported before. For instance, the ATF/CREB site of the tyrosine aminotransferase gene is cAMP inducible in FTO-2B cells and is largely constitutive in HeLa cells (30).

To determine whether the ATF/CREB site is required for virus induction of the HuIFN-β gene in HeLa cells, we analyzed the effects of point mutations in this region. We found that the sequence requirements for virus induction in HeLa cells are the same as in L929 cells while the effects are more significant (Fig. 6*D*; data not shown). Furthermore, as shown in Fig. 3*B*, addition of the ATF/CREB site to −91 IFN deletion completely restores its virus inducibility. Thus, the ATF/CREB site is required for virus induction in both cell types. Interestingly, the two mutations in the ATF/CREB site (−94G and −97T/−94G) that increase virus-induced expression without a significant change of the uninduced level in L929 cells increase both the basal and the induced levels of expression in HeLa cells (Fig. 6*D*). Thus, the transcription factors that are responsible for inducible activ-

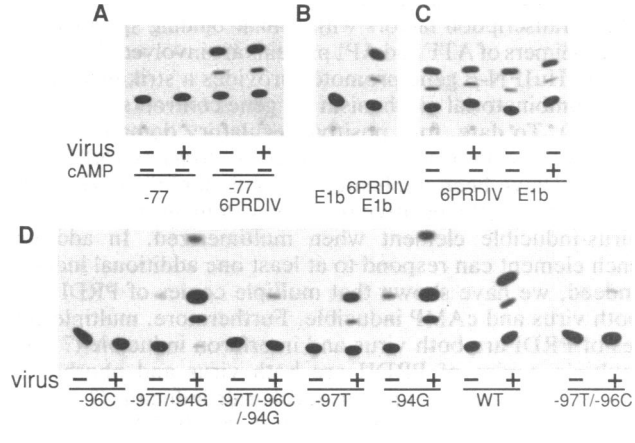

FIG. 6. Activity of PRDIV in HeLa cells. (*A*) Virus induction of −77 IFN CAT and six-PRDIV −77 IFN CAT constructs. (*B*) Comparison of the constitutive activity of E1b CAT with that of six-PRDIV E1b CAT. (*C*) Virus and cAMP induction of six-PRDIV E1b CAT construct. (*D*) CAT assay showing basal as well as induced activity of mutants of the ATF/CREB site in HeLa cells. Lanes: −, no induction; +, virus induced.

ity of PRDIV in L929 cells appear to have the same DNA recognition sequence as transcription factors that are responsible for the constitutive activity of PRDIV in HeLa cells.

We conclude that PRDIV acts as a constitutive transcription element in HeLa cells, which interacts synergistically with PRDI, PRDII, and PRDIII, the virus-inducible elements of the HuIFN-β promoter.

DISCUSSION

Our mutagenesis studies show that the PRDIV sequences required for virus induction in the −110 HuIFN-β promoter context correspond to an ATF/CREB site. We have also shown that multiple copies of PRDIV can confer both virus and cAMP inducibility on a minimal TATA promoter. However, multiple copies of the CRE can confer only cAMP, and not virus, inducibility on the minimal TATA promoter; similarly, multiple copies of the ATF/CREB site of the HuIFN-β promoter (−99 to −91) is poorly cAMP inducible and not virus inducible. These observations suggest that the ATF/CREB sites require additional flanking sequences for virus inducibility. These observations are consistent with the possibility that a protein responsible for virus induction of PRDIV both recognizes an ATF/CREB factor bound to DNA and interacts specifically with the flanking DNA sequences. A precedent for this possibility is the interaction between the P62/TCF protein and the serum-responsive factor when they are bound to the serum response element of the *Fos* gene promoter (31). Alternatively, it is possible that the ATF/CREB site directly overlaps a distinct regulatory sequence that is also required for virus induction.

We have shown that PRDIV is both virus and cAMP inducible. Previous studies showed that double-stranded RNA activates adenylate cyclase and leads to an increase in the level of cellular cAMP (32). These observations suggest that virus induction could involve the cAMP signal transduction pathway. It is interesting from this point of view that the NF-κB/IκB cytoplasmic complex can be activated *in vitro* by protein kinase A (33), and by double-stranded RNA or virus induction (8, 34–36). Although this observation suggests that cAMP could be involved in the virus induction of PRDII, we are unable to detect an effect of cAMP on the expression of a construct containing four copies of PRDII in L929 cells (unpublished data).

The possibility that ATF-2/CRE-BP1 is involved in HuIFN-β gene regulation is suggested by the correlation between *in vitro* binding of this protein and virus induction. However, it is equally likely that other members of the ATF/CREB family, other transcription factors with similar binding specificity, or heterodimers of ATF and AP1 proteins are involved (22, 37, 38).

The HuIFN-β gene promoter provides a striking example of a combinatorial mechanism for gene control (see ref. 2 for review). To date, four positive regulatory domains (PRDI–PRDIV) and two negative regulatory domains (NRDI and NRDII) have been implicated in regulation of the HuIFN-β gene. Each of the positive regulatory domains functions as a virus-inducible element when multimerized. In addition, each element can respond to at least one additional inducer. Indeed, we have shown that multiple copies of PRDIV are both virus and cAMP inducible. Furthermore, multiple copies of PRDI are both virus and interferon inducible (7), and multiple copies of PRDII are both virus and phorbol 12-myristate 13-acetate/lipopolysaccharide inducible (7, 11, 39). The combination of these elements in the HuIFN-β promoter is induced only by virus and not by any of the other inducers that act on individual elements.

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