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IRF-7, a New Interferon Regulatory Factor Associated with Epstein-Barr Virus Latency

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The Epstein-Barr virus (EBV) *Bam*HI Q promoter (Qp) is the only promoter used for the transcription of Epstein-Barr virus nuclear antigen 1 (EBNA-1) mRNA in cells in the most restricted (type I) latent infection state. However, Qp is inactive in type III latency. With the use of the yeast one-hybrid system, a new cellular gene has been identified that encodes proteins which bind to sequence in Qp. The deduced amino acid sequence of the gene has significant homology to the interferon regulatory factors (IRFs). This new gene and products including two splicing variants are designated IRF-7A, IRF-7B, and IRF-7C. The expression of IRF-7 is predominantly in spleen, thymus, and peripheral blood leukocytes (PBL). IRF-7 proteins were identified in primary PBL with specific antiserum against IRF-7B protein. IRF-7s can bind to interferon-stimulated response element (ISRE) sequence and repress transcriptional activation by both interferon and IRF-1. Additionally, a functional viral ISRE sequence, 5'-GCGAAAACGAAAGT-3', has been identified in Qp. Finally, the expression of IRF-7 is consistently high in type III latency cells and almost undetectable in type I latency, corresponding to the activity of endogenous Qp in these latency states and the ability of the IRF-7 proteins to repress Qp-reporter constructs. The identification of a functional viral ISRE and association of IRF-7 with type III latency may be relevant to the mechanism of regulation of Qp.

The malignant diseases associated with Epstein-Barr virus (EBV) are characterized by infection that is for the most part latent. Several types of latency are classified based on the pattern of viral protein expression. In a highly restricted form called type I latency, only EBNA1, which is required for replication of the EBV episome, is expressed and only the *Bam*HI Q promoter (Qp) is active for transcription of EBNA1 mRNA. However, in type III latency, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP-1, LMP-2A, and LMP-2B are expressed. Interestingly, Qp is inactive in type III latency, and another promoter is used for EBNA1 transcription (reviewed in references 23 and 37).

Qp transcriptional activity is controlled by both viral and cellular factors (5, 31, 39, 43, 44). A downstream element appears to be the final control point for Qp transcription. The sequence, the Q locus (see Fig. 5A), contains two binding sites for the EBNA1 protein, which binds and acts in an autoregulatory manner to repress Qp transcription (39, 44). However, E2F1 or an E2F-like molecule can displace the binding of EBNA1 through adjacent binding sites within the Q locus and overcome EBNA1-mediated repression of Qp (43).

An essential *cis*-acting element, whose mutation abolishes the constitutive activity of the promoter, has been identified in Qp (31, 44). Cloning of the products of cDNAs which can bind to this specific sequence uncovered a new member of the interferon (IFN) regulatory factor (IRF) family.

IRFs were first discovered through studies of regulation of an IFN gene (29). The IRF family includes IRF-1, IRF-2, IFN-stimulated gene factor 3 γ (ISGF-3 γ), IFN consensus sequence binding protein (ICSBP), human IRF-3, Pip/LSIRF/ICSAT, and chicken IRF-3 (cIRF-3), which is not the coun-

terpart to human IRF-3 (2, 10, 12, 16, 17, 28, 29, 50, 52, 56). Some IRFs are linked to IFN signal transduction; e.g., some of the IRFs are induced or activated by IFN, and at least two factors are involved in the regulation of an IFN gene (13, 14, 17, 22, 29, 50). However, IRFs have additional diverse functions, not necessarily linked to IFN, as regulators of cell growth and differentiation (12, 18, 19, 30, 46–48). The hallmark of this family is a conserved N-terminal DNA-binding domain with a five-tryptophan repeat (10, 50). In common to IRFs is the ability to bind to the core region of the IFN-stimulated response element (ISRE) found in the promoters of IFN-responsive genes.

The newly cloned gene and products including two splicing variants are designated IRF-7A, IRF-7B, and IRF-7C. Assays with reporter constructs raise the possibility that the IRF-7 proteins are implicated in IFN signal transduction at the transcriptional level. The discovery of IRF-7 has also led to the identification of a functional viral ISRE sequence in Qp. Finally shown here is an association of IRF-7 expression with type III latency in which IRF-7 expression is high. Since IRF-7 can repress Qp activity in reporter constructs, IRF-7 may contribute to rendering Qp inactive in type III latency.

MATERIALS AND METHODS

Cloning of IRF-7 with the use of the yeast one-hybrid system. Briefly, the double-stranded oligonucleotide with the sequence 5'-AGCTTTGCGAAACGTAAGTCGCTGCGACGCTTTTTCGAAACGAAAGTCGCGTACCGCTTTCGAAAACGAAAGTGTCT-3' was obtained by annealing two single-stranded oligonucleotides. The resulting oligonucleotide contains three copies of F7/8 sequence from EBV Qp and was subcloned into the *Xho*I site of pLR1-Δ1 (53) as well as the *Eco*RI site of pHR307a (55). The yeast strain EGY48 (a gift of R. Brent), harboring both plasmids, was transformed with DNA from the cDNA library made from EBV-immortalized B cells (11). Yeast cells with His⁺ β-Gal⁺ phenotypes were selected. The cDNA plasmids from His⁺ β-Gal⁺ yeast colonies were isolated; their ability to specifically activate 3xTf/8 sequences was confirmed by retransforming them into yeast. Then, two primers derived from the library vector plasmid were used as described previously (11). Primer A (5'-TAATACGACTCACTATAGGGAGACCACATGGATGAGTATATAACTATC

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ATTTC-3') contains a T7 promoter sequence placed in an appropriate position to allow the transcription of the cDNA insert by T7 RNA polymerase. Primer B is 5'-CTACCAGAATTCGGCATGCCGGTAGAGGTGTGGTCA-3'. The PCR product could then be directly added to a coupled transcription and translation system (TNT; Promega). The translation lysates were used in an electrophoretic mobility shift assay (EMSA) to test whether the protein generated could bind directly to the F7/8 sequence. Two positive clones, c2a and c5a, which encode proteins that can directly bind to the target sequence, were used for further analysis.

Sequence analysis of IRF-7 cDNA. The cDNA inserts of c2a and c5a were subcloned into the pBS+ vector at the *Bam*HI site to generate pBS-c2a and pBS-c5a, respectively. These two plasmids were subjected to nested deletional exonuclease III treatment (38). The set of deletion constructs from each end of the inserts were sequenced with M13 forward or M13 reverse primer. Both strands of cDNA were sequenced largely by the University of North Carolina sequencing facility with *Taq* DNA polymerase. For some ambiguous sequences, Sequenase (U.S. Biochemical) and single-stranded DNA were used to verify the sequences. A specific primer from clone c2a and primer B were used for PCR to isolate further 5' upstream sequence of the clones. The resulting PCR products from a human B-cell cDNA library were subcloned into the pBS+ vector. The three longest clones were sequenced, and identical sequences from these three clones were analyzed and deposited into GenBank.

Two Expressed Sequenced Tag (EST) database cDNA clones deposited by I.M.A.G.E. Consortium (25) were obtained and sequenced the same way as the c2a and c5a clones. The I.M.A.G.E. clone identification numbers for these clones are 172449 and 153389.

Plasmid construction. Both the F7 oligonucleotide, 5'-GATCGCTTTGCCGA AAACGAAAGTGC-3', and its complementary F8 oligonucleotide with a linker sequence 5'-GATC were annealed (F7/8) and subcloned into the *Bgl*II site of pA10CAT (24). The plasmid containing multiple (four) insertions of the F7/8 fragment was named pA10-F7/8. The c5a and c2a cDNA inserts were subcloned into the *Bam*HI site of the pcDNA3 vector to generate the IRF-7A and IRF-7B expression plasmids pcDNA-IRF-7A and pcDNA-IRF-7B, respectively. The *Msc*I-*Not*I fragment of clone 172449 was subcloned into pcDNA3 at the *Eco*RV and *Not*I sites and named pcDNA-IRF-7C. The *Msc*I-*Sma*I fragment of clone 172449 was subcloned into the *Eco*RV site of pcDNA3 and named pcDNA-IRF-7C.1. pcDNA-IRF-7C.1 contains the complete open reading frame (ORF) for IRF-7C, but lacks the 3' end of the clone containing the complete IRF-7C.2 ORF (see Fig. 1A). The IRF-1 cDNA fragment from SV-1SGF2 (35) was transferred into the pBS+ vector (pBS-IRF-1) for in vitro transcription and translation.

In vitro transcription and translation. The proteins were made with the TNT coupled transcription and translation kit (Promega) essentially as specified by the manufacturer. Plasmids pcDNA-IRF-7A, pcDNA-IRF-7B, pcDNA-IRF-7C, and pcDNA-IRF-7C.1 were used for translation of IRF-7 proteins, and pBS-IRF-1 was used for IRF-1.

Production and purification of antibody against IRF-7. The *Bgl*II fragment of c2a (IRF-7B) was subcloned into the *Bam*HI site of the pET-28a (+) vector (Novagen). The protein was expressed in the *Escherichia coli* host BL21(DE3) with a polyhistidine tag at the N terminus. Protein expression was induced by treatment with 0.8 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 h. *E. coli* was pelleted and prepared for purification under denaturing conditions with Ni-nitrilotriacetic acid resin as specified by the manufacturer (Qiagen). The purified protein was sent to Cocalico Biological, Inc., for antibody production in a rabbit. The antiserum was diluted 1:10 and used for EMSA. However, for Western blot analysis, the antiserum was affinity purified by a standard method (38).

Northern blot analysis. The multiple-human-tissue mRNA blot was purchased from Clontech. The IRF-7A (c5a) fragment was labeled with [α -³²P]dATP and [α -³²P]dCTP by random priming with Klenow enzyme (38). The subsequent hybridization and washing were done as recommended by the manufacturer.

PBL. The fresh peripheral blood leukocytes (PBL) from healthy donors were gifts from S. Haskill, UNC-Lineberger Comprehensive Cancer Center.

Western Blot analysis with enhanced chemiluminescence. Separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed standard methods. After the proteins were transferred to a nitrocellulose or Immobilon membrane, the membrane was blocked with 5% nonfat dry milk in TBST (50 mM Tris [pH 7.5], 200 mM NaCl, 0.05% Tween 20) at room temperature for 10 min. It was then washed briefly with water and incubated with the first antibody in 5% milk in TBST for 1 h at room temperature or overnight at 4°C. After being washed with TBST three times (10 min each), the membrane was incubated with the secondary antibody at room temperature for 1 h. It was then washed three times with TBST as before, treated with enhanced chemiluminescence detection reagents (Amersham), and exposed to Kodak XAR-5 film.

EMSA. The DNA probes were generated by annealing oligonucleotides and filling in any single-stranded overhang with Klenow enzyme. EMSA was performed essentially as described previously (26). In vitro-translated reticulocyte lysates (5 μ l) were incubated for 20 min at room temperature with 20,000 to 50,000 cpm of labeled probe in a volume of 12.5 μ l containing 20 mM HEPES (pH 7.9), 1 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 320 μ g of poly(dI-dC)-poly(dI-dC), and 4% Ficoll-400. The samples were separated on a

preelectrophoresed 4.8% polyacrylamide gel in 20 mM Tris-borate-EDTA (TBE) buffer. After electrophoresis, the gels were dried and subjected to autoradiography. The conditions for the competition assay between IRF-1 and IRF-7s were essentially the same, except that less probe was used (3,000 to 4,000 cpm). When antiserum was needed, 1 μ l was added to the reaction mixture. The consensus ISRE oligonucleotide was synthesized as specified in a previous report (22). AP-1 and SP-1 competitors were purchased from Promega.

Cell culture, transient transfection, and CAT assay. DG75 is an EBV-negative Burkitt's lymphoma cell line (3); Sav I and Sav III, and Kem I and Kem III are paired EBV-positive Burkitt's lymphoma cell lines that differ only in their latent infection state (gifts from J. Sample [32]). All cells were maintained in RPMI 1640 plus 10% fetal bovine serum. Electroporation was used for transfection as described previously (43). IFNs were added at a final concentration of 500 IU/ml about 30 min after transfection. Cell lysates were prepared after 36 to 48 h. The chloramphenicol acetyltransferase (CAT) assays were performed by standard methods (15) and analyzed on a Molecular Dynamics PhosphorImager. The results represented an average of at least three independent transfections; two batches of plasmid preparations were used.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to GenBank under accession no. U53830 to U53832.

RESULTS

Cloning of IRF-7 by the yeast one-hybrid system. A *cis*-acting region needed for constitutive activity of Qp was localized to a 21-bp sequence through its protein-binding activity (F7/8, nucleotides [nt] 62399 to 62419 of EBV B95-8 [see Fig. 5A]). A point mutation in this region abolished both specific DNA-binding and promoter activity (44). The F7/8 sequence (three insertions) was used in a yeast one-hybrid system, similar to that previously published (20, 51) (see Materials and Methods), to identify any cellular protein(s) that bound to the F7/8 sequence. Two positive cDNA clones, c2a and c5a, were selected for sequencing analysis because they encoded proteins that could bind to the target sequence (F7/8) directly in EMSA (data not shown). The two clones have identical sequences, except that c5a is 87 nt longer than c2a. The c2a clone is apparently a splicing variant of c5a and lacks 29 amino acids (aa) in its ORF (Fig. 1A and B; the c5a and c2a products have been renamed IRF-7A and IRF-7B, as explained below). Since the 5' upstream sequence of both clones did not contain an in-frame stop codon, PCR was used to isolate further 5' upstream sequence of the cDNAs, which disclosed an in-frame stop codon. Thus, translational initiation codons were positively identified for both the c2a and c5a clones. A database search with the final deduced amino acid sequence revealed that these two cDNAs encode novel proteins most closely related to the IRF family. Both clones showed 29 to 52% identity to various members of the IRFs in their conserved N-terminal DNA-binding domain (Fig. 1C). The IRF member with highest homology to our clone is cIRF-3. The c5a clone and cIRF-3 have 42% overall identity and 52% identity in the N-terminal regions. This new gene and its products are designated IRF-7A (c5a clone) and IRF-7B (c2a clone). A database search with the nucleotide sequence showed that the 3' end of the cDNA matched the sequences of two clones in the EST database (98% identity in a ca. 300-nt region). Sequence analysis disclosed that these two clones are identical in their 3'-end sequence; however, one of them does not have a complete 5'-end sequence. Both EST clones also appear to be a splicing variant of IRF-7A and lack 227 nt in the middle of the cDNA. This splicing actually changes the original reading frame so that a smaller protein (164 aa) may be generated, which is designated IRF-7C (Fig. 1A and B).

There are two cryptic ORFs in both IRF-7A and IRF-7B cDNAs that are absent in IRF-7C cDNA. These ORFs are in an alternative reading frame from IRF-7A and IRF-7B. The additional ORF in IRF-7A cDNA has 200 aa, and the cryptic ORF in IRF-7B cDNA has 171 aa because 29 aa in the middle

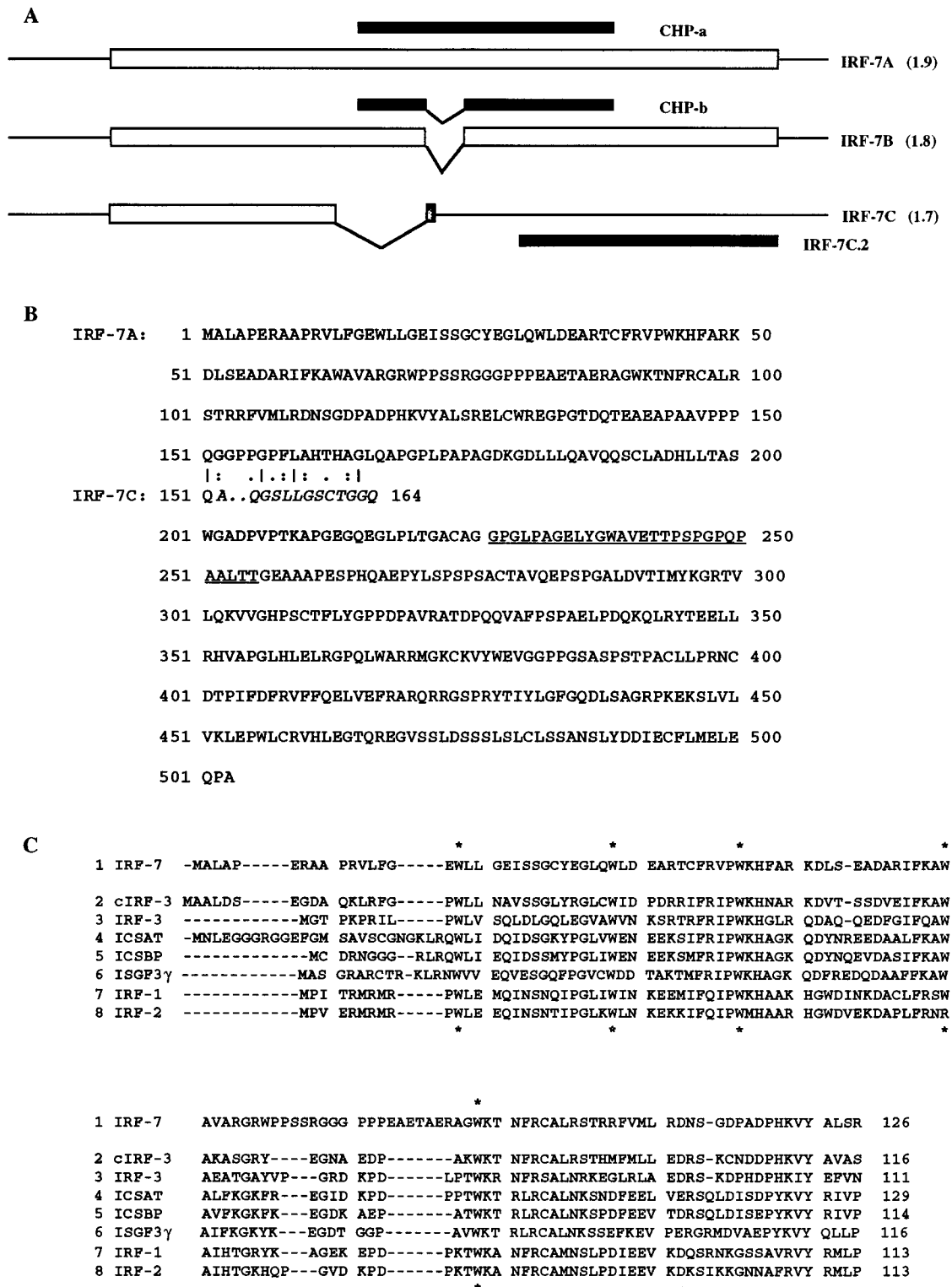


FIG. 1. cDNA and protein structures of IRF-7s. (A) The ORFs are represented by open and solid bars. The open bars represent the ORFs confirmed as encoding proteins. IRF-7C has a 13-aa sequence difference from IRF-7A in its C terminus (Fig. 1B), indicated by the shaded bar. The solid bars indicate the unconfirmed ORFs. The lengths of cDNAs (in kilobases) are shown in parentheses. (B) The primary sequence of IRF-7s deduced from the nucleotide sequence of cDNAs. The deduced amino acid sequence of IRF-7A is shown. The underlined sequence is that missing in IRF-7B. The first 151-aa sequence of IRF-7C is identical to that of IRF-7A and IRF-7B. The last 13 aa of IRF-7C are different and are shown in italics. The sequence homology between the 13 aa of IRF-7C and of IRF-7A and IRF-7B is also shown. (C) Alignment of the N-terminal region of IRF-7 with other members of the IRF family. Gaps have been introduced to allow the best alignment. The conserved tryptophans are indicated by asterisks.

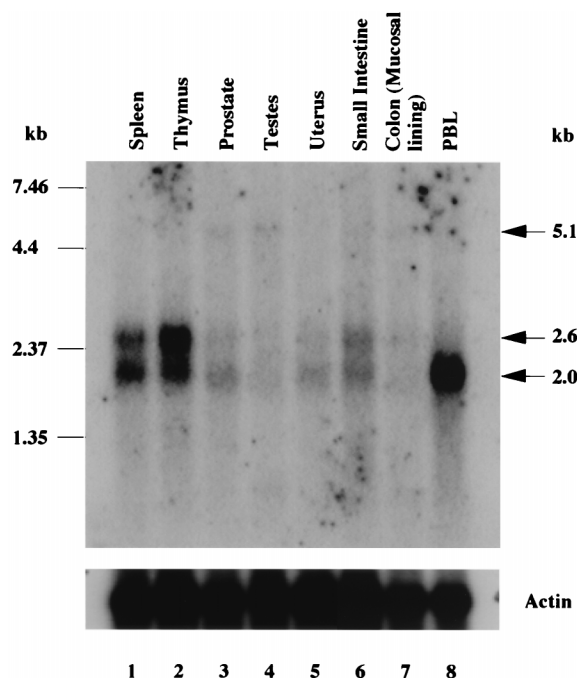


FIG. 2. Expression of IRF-7 mRNA in various human tissues. Lanes 1 to 8 contain 2 μ g of mRNA each from spleen, thymus, prostate, testes, uterus, small intestine, colon (mucosal lining), and PBL, respectively, which had been separated and blotted (Clontech). The blot was examined by Northern analysis with labeled IRF-7A cDNA as a probe. The positions of RNA standards (in kilobases) are indicated on the left. The positive signals, with sizes, are indicated by arrows on the right. The bottom panel indicates the level of actin mRNA as control.

is missed due to splicing. A sequence homology search disclosed that these hypothetical proteins have low homology (24% identity) to human type II collagen protein in a 120-aa region (6). Since some proteins can be translated from different ORFs within the same mRNA (36), these hypothetical proteins are designated collagen homolog protein a (CHP-a) and CHP-b, respectively (Fig. 1A).

IRF-7C cDNA also contains an additional ORF, located in the 3' end of the cDNA; its amino acid sequence is identical to aa 294 to 503 of IRF-7A, presuming that the methionine codon at aa 294 (of IRF-7A) could be used as a translational initiation codon (Fig. 1A). This hypothetical protein was designated IRF-7C.2. A region in IRF-7C.2, which is also present in IRF-7A and IRF-7B, has some homology to the interaction domain of ISGF-3 γ with Stat1 and Stat2 (50). Whether CHPs and IRF-7C.2 can be translated from their mRNAs is unknown.

With labeled IRF-7A cDNA as the probe, human genomic DNA from both DG75 and HeLa cells digested by *Bam*HI or *Hind*III disclosed a single predominant band in Southern blot analysis (data not shown). The results suggest that the human genome contains only one copy of the IRF-7 gene. Therefore, IRF-7B and IRF-7C are most likely to be splicing variants of IRF-7A rather than being derived from different genes.

Tissue distribution of IRF-7 mRNA. Northern analysis of a blot of multiple human tissue mRNAs (Clontech) with labeled IRF-7A cDNA as a probe showed that IRF-7 mRNAs are expressed predominately in spleen, thymus, and PBL (Fig. 2, lanes 1, 2, and 8). However, almost all the other tissues examined showed a low level of expression. There were two major bands, of 2 and 2.6 kb respectively, that hybridized to IRF-7A probe. Interestingly, the 2.6-kb band appeared strong in both

spleen and thymus; however, it was weak in PBL (compare lanes 1 and 2 with lane 8). The identity and significance of these bands are unknown, and it is hard to distinguish the various mRNAs of IRF-7. However, the 2-kb mRNA should contain at least some IRF-7A mRNA since IRF-7A is the major form of IRF-7 proteins identified in PBL (Fig. 3A). Besides the two major bands, an additional weak band of 5.1 kb appeared in every tissue examined. This might be an additional splicing variant, an unprocessed RNA, or a nonspecific band.

Identification of IRF-7 protein in primary cells. To confirm that the cDNA clones actually encode the predicted proteins, cDNA fragments were cloned into an expression vector, pcDNA3. Antibody against IRF-7 was generated by inoculating purified full-length IRF-7B protein into rabbits (see Materials and Methods). The antiserum was affinity purified before being subjected to Western blot analysis (38). The results in Fig. 3 clearly show that IRF-7s are expressed and identified from these cDNAs in cells (lanes 1 to 4). The sizes of the proteins detected (69, 67, and 23 kDa for IRF-7A, IRF-7B, and IRF-7C, respectively) were close to the predicted sizes and also matched the sizes of the in vitro-translated proteins (data not shown). From the tissue distribution of mRNA by Northern blot analysis (Fig. 2), the IRF-7 gene should be expressed in PBLs. Six normal PBLs (three of them are shown) obtained from healthy donors disclosed the presence of IRF-7A protein (Fig. 3A, lanes 5 to 7). However, the expression of IRF-7B and IRF-7C is uncertain. A weak band corresponding to the plasmid-expressed IRF-7B and IRF-7C can be detected (data not

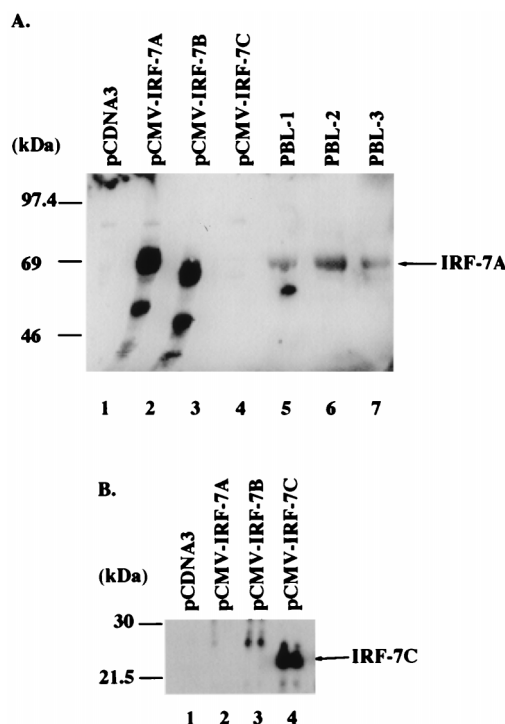


FIG. 3. Identification of IRF-7 proteins in primary PBL. (A) Protein samples were separated by SDS-8% PAGE and subjected to Western blot analysis with antibody against IRF-7B. Lanes: 1 to 4, DG75 cells transfected by pcDNA3 (lane 1), pcDNA-IRF-7A (lane 2), pcDNA-IRF-7B (lane 3), and pcDNA-IRF-7C (lane 4); 5 to 7, PBLs from three normal healthy donors. The molecular mass standards (Rainbow markers; Amersham) are indicated on the left in kilodaltons. (B) Conditions are identical to those in panel A, except that SDS-12% PAGE was used to identify IRF-7C. Lanes 1 to 4 are the same as in panel A.

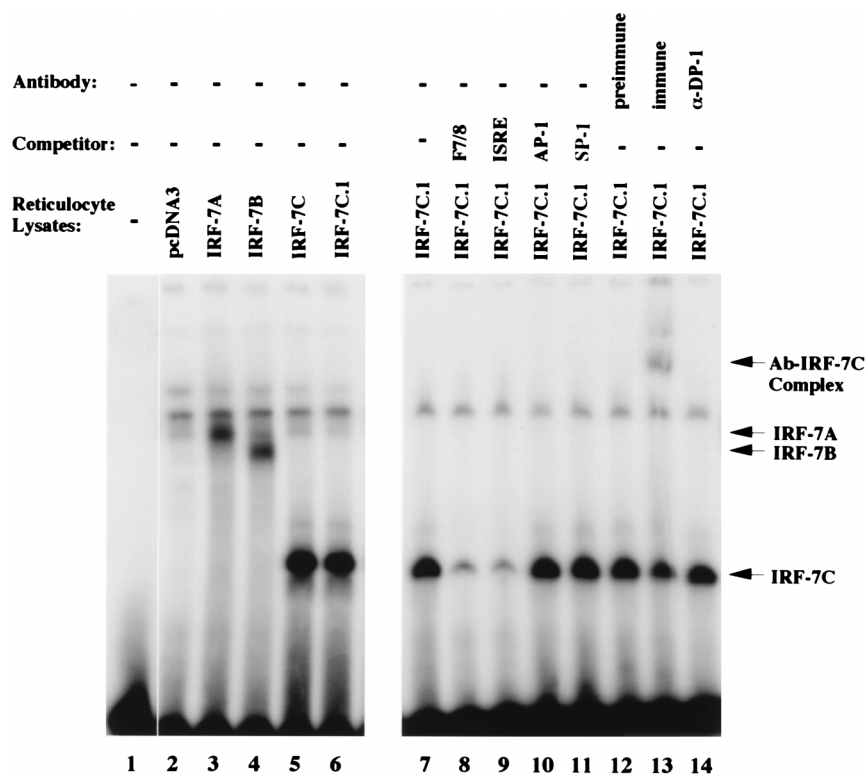


FIG. 4. IRF-7s are DNA-binding proteins. The probe was F7/8 labelled with [α - 32 P]dCTP. Cold competitors were all added at a 100-fold molar excess over hot probe (F7/8). The lanes labeled preimmune and immune contain preimmune and immune sera for intact IRF-7B protein. Lanes: 1, free probe; 2 to 5, reticulocyte lysates containing in vitro-translated proteins from plasmids pcDNA3, pcDNA-IRF-7A, pcDNA-IRF-7B, pcDNA-IRF-7C, respectively, used for EMSA; 6 to 14, reticulocyte lysate containing in vitro-translated protein from pcDNA3-IRF-7C.1. F7/8 and ISRE sequence from ISG15 were used as cold competitors in lanes 8 and 9, and AP-1 and SP-1 were used in lanes 10 and 11. Preimmune serum was used in lane 12, and immune serum was used in lane 13. Nonrelevant rabbit polyclonal antibody against DP-1 (Santa Cruz) was used in lane 14. The arrows denote IRF-7-specific bands.

shown). Apparently, IRF-7A is the predominant form in PBL. Several fast-migrating proteins are generated with IRF-7A and IRF-7B expression constructs (lanes 2 and 3). These forms possibly originate from the usage of internal methionine codons for translational initiation. Other explanations include additional splicing of mRNA and proteolytic processing of the intact proteins. Interestingly, PBLs, which have strong expression of IRF-7A, did not show these additional bands.

IRF-7 proteins are DNA-binding proteins. The results of the yeast one-hybrid screening indicated that IRF-7 could bind to DNA in vivo. EMSA was then used to test the DNA-binding properties of intact IRF-7 in vitro. IRF-7s were translated in vitro, and their expression was confirmed by Western blot analysis (data not shown). These lysates were used for EMSA. As shown in Fig. 4, specific bands appeared when IRF-7s were used for EMSA (lanes 3 to 6). These bands were specific for the following reasons. (i) They disappeared in the presence of an excess of cold competitors, such as F7/8 and consensus ISRE from the IFN-stimulated gene 15 (ISG 15) promoter (Fig. 4, lanes 8 and 9), but nonspecific competitors, such as AP-1 and SP-1, had no effect (lanes 10 and 11). The consensus ISRE was used as a competitor because a similar element was found in F7/8 and subsequently proven to function as an ISRE (see below and Fig. 5 for details). (ii) DNA-binding activity was not affected when preimmune serum and nonrelevant antibody (anti-DP-1) were used (Fig. 4, lanes 12 and 14); however, specific IRF-7 antibody could supershift the IRF-7C-DNA complex (lanes 13). The DNA-binding activity of IRF-7A and

IRF-7B proteins was also specific (data not shown). Essentially identical results were obtained with consensus ISRE sequence from ISG 15 as a probe (data not shown). Thus, these forms of IRF-7s are DNA-binding proteins that bind specifically to ISRE sequence both in vivo (yeast one-hybrid) and in vitro (EMSA). Furthermore, IRF-7C, which has only 164 aa, can bind to the ISRE sequence, indicating that the DNA-binding domain of IRF-7, similar to other IRFs, is localized in the N-terminal region (Fig. 1A).

EBV Qp has a functional ISRE site. After the cloning of IRF-7, an ISRE sequence was identified in Qp: 5'-GCGAAA ACGAAAGT-3' (nt 62404-62417 of the EBV B95-8 strain [Fig. 5A]). This is identical to the consensus ISRE sequence, 5'-(A/G)NGAAANNGAAACT-3' (8), except at the underlined position, where the conserved C is replaced by a G in Qp. The same C-to-G replacement has been found in the ISRE of the promoter of guanylate-binding protein (9). The obvious functional experiment was to test whether Qp can be activated upon IFN treatment. However, the full-length Qp reporter construct did not respond to IFN- α , IFN- β , or IFN- γ treatment (data not shown). This result might be due to negative regulatory elements in the promoter or the cell lines tested. Next, four copies of F7/8, which contains the putative ISRE sequence, were placed in front of an enhancerless promoter-reporter construct, pA10CAT. The resulting construct, pA10-F7/8 (Fig. 5A), was introduced into DG75 cells by electroporation. Both IFN- α and IFN- β could transactivate the reporter construct eightfold (Fig. 5B, lanes 4 and 5). However, IFN- γ had a limited effect (lane 6). Based on the responsiveness to

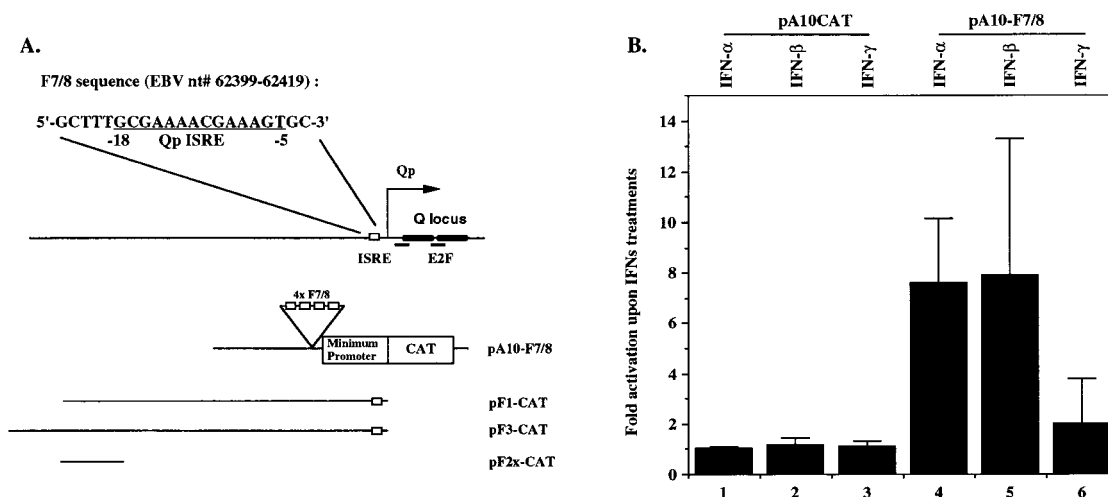


FIG. 5. Stimulation of an element in Qp by IFN. (A) Schematic representation of Qp promoter constructs used in transient-transfection assays (modified from reference 43). The RNA start site for Qp is indicated by an arrow (41). The F7/8 sequence, ISRE (open square), and its relative coordinates to the Qp start site, as well as promoter constructs used for Fig. 6C, are shown. The Q-locus (solid oval) and E2F1-binding sites (lines) are also shown. (B) EBV Qp contains an ISRE. DG75 cells were transfected with pA10CAT or pA10-F7/8. The transfected cells were divided equally into two wells of a six-well plate, and IFN- α , IFN- β , or IFN- γ was added in one well. Cell lysates were used for the CAT assay by standard procedures and analyzed on a Molecular Dynamics PhosphorImager. The results are expressed as fold activation upon IFN treatment. The final concentration of all IFNs was 500 IU/ml. Standard error bars are shown.

both IFN- α and IFN- β , as well as the sequence similarity to consensus ISRE, we concluded that Qp has a functional ISRE sequence.

Expression of IRF-7 is associated with EBV latency. Sav I and Sav III, as well as Kem I and Kem III, are sister Burkitt's lymphoma lines, each derived from a single parental cell line. The paired lines differ only in their types of latency. Whether IRF-7 is associated with EBV latency was tested by Western blot analysis with IRF-7 antibody. As shown in Fig. 6B, IRF-7A is expressed at a much higher level in type III cells (Fig. 6B, lanes 2 and 4) than in type I cells (lanes 1 and 3). However, the IRF-1 levels were essentially the same in the type I and III cells (data not shown). Latency types were confirmed by detection of EBNA1, EBNA2, and LMP1 proteins by Western blot analysis with specific antibodies (data not shown). IRF-7A was also expressed at a higher level in SFC-4 and CB95 cells, which are type III (33), than in Akata type I cells (reference 45 and data not shown). Therefore, without exception, in all the cell lines tested, the expression of IRF-7 is greater in type III than in type I latency.

IRF-7 proteins can repress the activity of Qp reporter constructs. The function of IRF-7 in the regulation of Qp was studied in cotransfection experiments with DG75 cells, chosen because these cells contain trace amounts of endogenous IRF-7s (Fig. 3, lanes 1). Cotransfection of IRF-7-expression plasmids with pF1-CAT, a Qp reporter construct containing the ISRE sequence (43) (Fig. 5A), resulted in decreases in the basal activity of Qp (Fig. 6C, lanes 2 to 5). However, IRF-1 can activate this reporter construct (less than twofold [data not shown]), confirming a recent report (40). A similar result was obtained when pF3-CAT (Fig. 5A), another Qp reporter construct containing ISRE sequence, was used for this experiment (data not shown). At the same time, different IRF-7 expression plasmids were cotransfected with pF2x-CAT (Fig. 5A), which does not contain the ISRE sequence, or cotransfected with a Rous sarcoma virus CAT construct into DG75 cells. IRF-7s repressed neither pF2x-CAT nor a Rous sarcoma virus reporter construct (data not shown), indicating that the repression of Qp by IRF-7s is specific. Thus, three forms of IRF-7 repressed Qp activity by 45 to 70%.

IRF-7 proteins repress IFN- α -activated reporters through the ISRE. Since some IRFs are regulators of IFN signal transduction, the effect of IRF-7 on the activation of promoter constructs by IFN was tested. The three forms of IRF-7 (A, B, and C) could significantly reduce IFN- α -induced activation of the Qp ISRE construct (Fig. 7A, lanes 2 to 4). Activation by IFN- α of another reporter construct, 15K ISRE(1), with a single copy of the ISRE sequence from the cellular ISG 15 promoter placed in front of human immunodeficiency virus (HIV) long terminal repeat promoter sequence (HIV-CAT) (35), could also be repressed (Fig. 7B). The vector constructs, HIV-CAT and pA10CAT, could not be activated by IFN- α (data not shown). Therefore, the activation by IFN- α and subsequent repression by IRF-7s must be through the ISRE sequence.

IRF-7 proteins repress transactivation by IRF-1 through the ISRE. Since IRF-2, ICSAT, and ICSBP can inhibit the activation of certain reporter constructs by IRF-1, we tested whether IRF-7 could inhibit IRF-1-induced transactivation. Cotransfection of IRF-1 and 15 K ISRE(4), which has four copies of ISRE sequence from the ISG15 promoter in front of the HIV minimal promoter (35), led to about a ninefold activation (Fig. 8, lanes 1 and 2). However, with coexpression of IRF-7s, activation of the reporter gene by IRF-1 was reduced significantly (lanes 3 to 6), similar to the inhibitory effect of IRF-2, which is a known repressor of IRF-1 (lane 7). Therefore, IRF-7s can repress transactivation by IRF-1. The vector construct, HIV-CAT, could not be activated by IRF-1 (data not shown). Hence, the activation by IRF1 and subsequent repression by IRF-7s must be through the ISRE sequence.

IRF-7 proteins can compete with IRF-1 for the ISRE site. Since IRF-1 and IRF-7s all can bind to ISRE sequence, the different factors may compete for binding to ISRE and provide the mechanism through which IRF-7s repress transactivation by IRF-1. As a general test of such a possibility, graded amounts of in vitro-translated IRF-7A or IRF-7C lysates were mixed with a fixed amount of in vitro-translated IRF-1, and EMSA was performed. The binding of IRF-1 to ISRE sequence gradually decreased as the amount of IRF-7 was increased (Fig. 9A, lanes 5 to 7; Fig. 9B, lanes 6 to 8). Non-

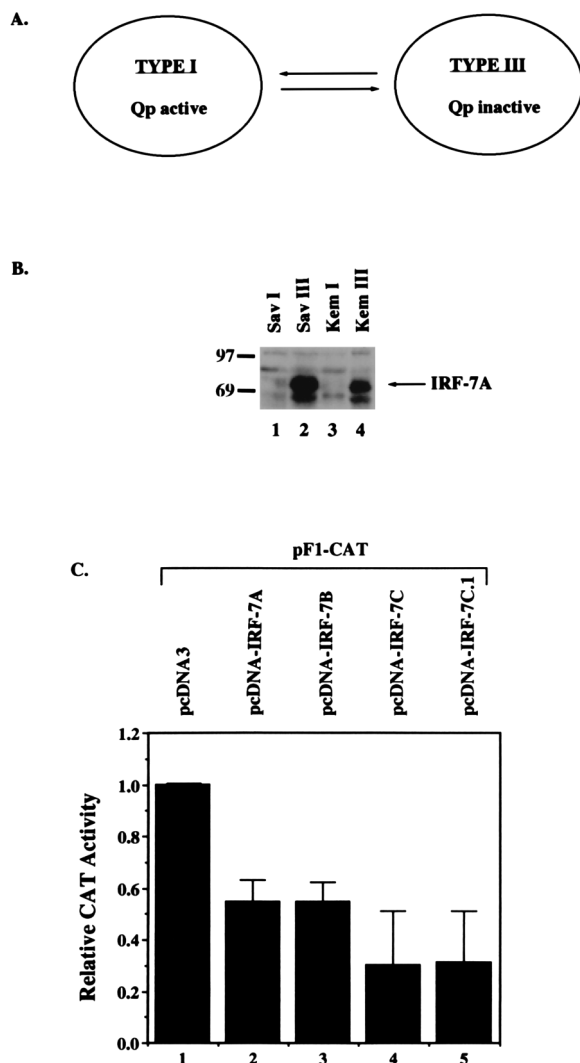


FIG. 6. Correlation between expression of IRF-7, type of latency, and repression of Qp. (A) Schematic diagram of Qp status and EBV latency. (B) IRF-7A is associated with type III latency. Equal amounts of protein lysates were electrophoresed by SDS-10% PAGE and stained with Ponceau S Red after transfer of protein to the membrane. Western blot analysis with IRF-7 antibody was performed. The identity of IRF-7A was determined by comparison with plasmid-expressed IRF-7A and IRF-7B. Lanes: 1 to 4, cell lysates from Sav I, Sav III, Kem I, and Kem III, respectively. The molecular mass standards (Rainbow markers) are indicated on the left in kilodaltons. (C) Repression of Qp activity by IRF-7s. DG75 cells were transfected with pF1-CAT reporter construct and pcDNA3, pcDNA-IRF-7A, pcDNA-IRF-7B, pcDNA-IRF-7C, and pcDNA-IRF-7C.1. Cell lysates were used for the CAT assays. The results are expressed as CAT activity relative to the vector control. Standard error bars are shown.

relevant protein, the *in vitro*-translated EBV BZLF-1 protein, did not produce such an effect (Fig. 9A, lane 8; Fig. 9B, lane 9). Therefore, both IRF-7A and IRF-7C can compete with IRF-1 for binding to ISRE. IRF-7B was not tested because it migrates close to IRF-1 in EMSA (data not shown).

DISCUSSION

IRF-7 and EBV latency. Our data strongly point to the relevance of IRF-7 as a repressor of Qp in type III latency: (i) the expression of IRF-7 is consistently high in type III cells, where

Qp is inactive, and low in type I cells, where Qp is active (Fig. 6A and B); (ii) overexpression of IRF-7 can repress the constitutive activity of Qp-reporter constructs (Fig. 6C); (iii) IRF-7 was isolated through a yeast one-hybrid system with the Qp ISRE sequence as bait, indicating IRF-7 can bind to Qp *in vivo*; and (iv) *in vitro*-translated IRF-7s can bind to Qp, as shown by EMSA with Qp ISRE as a probe (Fig. 4).

Qp is obviously carefully regulated. Autorepression by its viral product, EBNA-1, and release of this repression by E2F are unique features of Qp regulation (39, 43, 44). However, perhaps the most intriguing aspect of Qp is how it is rendered inactive selectively. Selective control of the promoter appears to be cellularly based. The data in this paper suggest that IRF-7 may act as a repressor but that its availability depends on the latency cell type, namely, type III. Inactivation of Qp might be the result of composite effects of IRF-7 and possibly other IRFs, as well as other repressors, such as EBNA1.

It is interesting that Qp has a functional ISRE, which suggests that other IRFs may also have the potential to bind to and provide Qp with a spectrum of regulatory possibilities. This idea is supported by a recent report that IRF-1 and IRF-2 are responsible for the constitutive activity of Qp (40), as well as our own data that IRF-1 can activate and IRF-7s can repress Qp activity.

Furthermore, EBNA2 (expressed in type III but not type I latency) has an anti-IFN effect. Burkitt's lymphoma cells expressing EBNA2 protein can override the antiproliferative effect of IFN and reduce or abolish the expression of IFN-stimulated genes (1, 21). The resistance of type III cells to the antiproliferative effect of IFN may be related to high levels of IRF-7 expression in such cells (1, 21), since IRF-7 is a negative regulator of IFN and IRF-1 responsiveness. However, this point must be vigorously tested.

Finally, the presence of a functional ISRE in Qp suggests that Qp can be activated by IFN; however, IFN does not activate intact Qp in CAT assays and in latently infected cells (reference 40 and data not shown). Two possible explanations for this result are that (i) EBNA1 can repress Qp activity (autoregulation), making the activation of Qp undetectable, and (ii) EBNA2 has an anti-IFN effect, making the activation of Qp in type III cells by IFN more difficult. The best time to detect Qp activation by IFN might be in primary infection, where EBNA1 and EBNA2 are synthesized at much lower levels. We are working on EBV infection of primary B cells to see if IFN can activate Qp.

Structure and function of IRF-7. The tissue distribution of IRF-7 mRNA suggests that tissue-specific splicing is occurring. IRF-7 is expressed predominantly in lymphoid tissues. However, there are two major bands in the spleen and thymus (2.6 and 2 kb) and only one in PBL (2 kb) (Fig. 2). IRF-7C is somewhat reminiscent of processed IRF-2 (7, 34). After viral infection, IRF-2 can be processed by a proteolysis, resulting in an approximately 160-aa N-terminal peptide which can also bind to DNA. The ability of processed IRF-2 to function as a repressor is significantly less than that of intact IRF-2 (34). However, other studies show that the truncated versions of IRF-2 repress reporter genes more efficiently than full-length IRF-2 does (27, 54). Since IRF-2 is a negative regulator and an antagonist to IRF-1, processing of IRF-2 is thought to be involved in the IFN-regulated genes in which the ratio of IRF-1 to IRF-2 can affect the level of transcription. IRF-7C, which is close in size (164 aa) to the truncated version of IRF-2 (~160 aa), is generated through splicing rather than proteolytic digestion.

The sequence homology of IRF-7 to other IRFs suggests

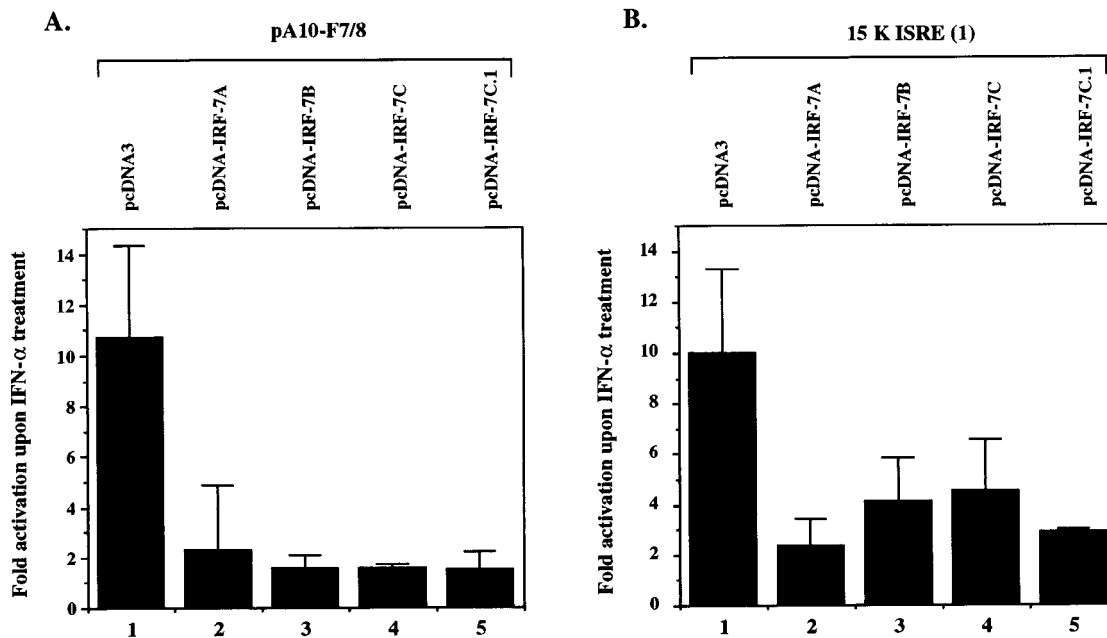


FIG. 7. Overexpression of IRF-7s inhibits IFN- α -induced activation. The experiments were done essentially as described in the legend to Fig. 5. The results are expressed as fold activation upon treatment with IFN- α (500 IU/ml). (A) pA10-F7/8 was cotransfected with pcDNA3 vector, pcDNA-IRF-7A, pcDNA-IRF-7B, pcDNA-IRF-7C, or pcDNA-IRF-7C.1. (B) Conditions are identical to those for panel A, except that the reporter construct 15K ISRE(1) was used. Standard error bars are shown.

that IRF-7 might function in the regulation of IFN stimulation. The evidence in favor of such a role is strong. First, the expression of IRF-7s inhibits the IFN-activated ISRE reporter constructs, including the newly identified ISRE from EBV Qp, as well as an ISRE from a well-studied cellular promoter, ISG15. Second, IRF-7s can negatively regulate transactivation of IRF-1 through an ISRE. Since ISRE is a highly conserved sequence and is present in the promoters of almost all IFN-regulated early genes, it is tempting to speculate that IRF-7s may be general regulators of IFN-regulated genes.

The structure of IRF-7C suggests that it might be a dominant negative regulator for IRF-7A and IRF-7B (Fig. 1A). However, all three IRF-7s act as negative regulators of IFN responses and transactivation by IRF-1, although the degree of repression differs. As with other IRFs, IRF-7 may have its own specific cellular function(s) and IRF-7C might be a dominant negative regulator for IRF-7A and IRF-7B in the function(s) related to the C-terminal region of the molecule (Fig. 1A).

Since the pcDNA-IRF-7C.1 expression plasmid, which contains only the IRF-7C ORF and lacks the CHPs and IRF-7C.2 ORFs (Fig. 1A), has an effect similar to IRF-7A, IRF-7B, and IRF-7C in regulating IFN responses and Qp, CHPs and IRF-7C.2 are most probably not involved in the regulation of IFN responses and Qp.

Knowledge of the mechanism of repression by IRF-7 is limited, except that IRF-7s can compete with IRF-1 for binding to the ISRE sequence in vitro (Fig. 9); however, it is still not clear how effectively IRF-7 can compete with IRF-1 in vivo. It also quite possible that IRF-7 is able to interact with and neutralize an activator, such as IRF-1 and/or ISGF3. The C-terminal regions of IRF-7A and IRF-7B have some homology to the interaction domain of ISGF-3 γ with Stat1 and Stat2 (49), which makes an interaction between Stat1 and Stat2 (components of the ISGF3 complex) and IRF-7 plausible. It has also

been reported that members of the IRF family can interact with each other (4, 42).

So far, several members of the IRF family are known to be negative regulators of IFN-regulated genes, including IRF-2, ICSP, and Pip/LSIRF/ICSAT, to which may be added the newly identified IRF-7. These apparently redundant negative

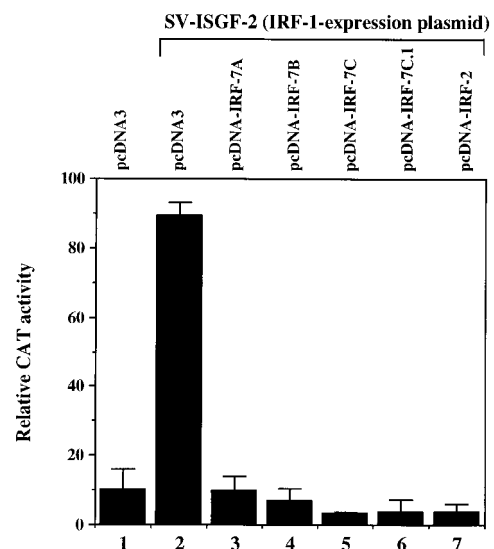


FIG. 8. Overexpression of IRF-7s inhibits transactivation by IRF-1. DG75 cells were transfected with the reporter construct 15K ISRE(4), and IRF-1, as well as IRF-7 expression plasmids. Lanes: 1, pcDNA-3 vector alone; 2 to 7, SV-ISGF-2, the IRF-1 expression plasmid, plus pcDNA3 vector, pcDNA-IRF-7A, pcDNA-IRF-7B, pcDNA-IRF-7C, pcDNA-IRF-7C.1, or pcDNA-IRF-2, respectively, were cotransfected. CAT assay results are expressed as CAT activity relative to the vector control. Standard error bars are shown.

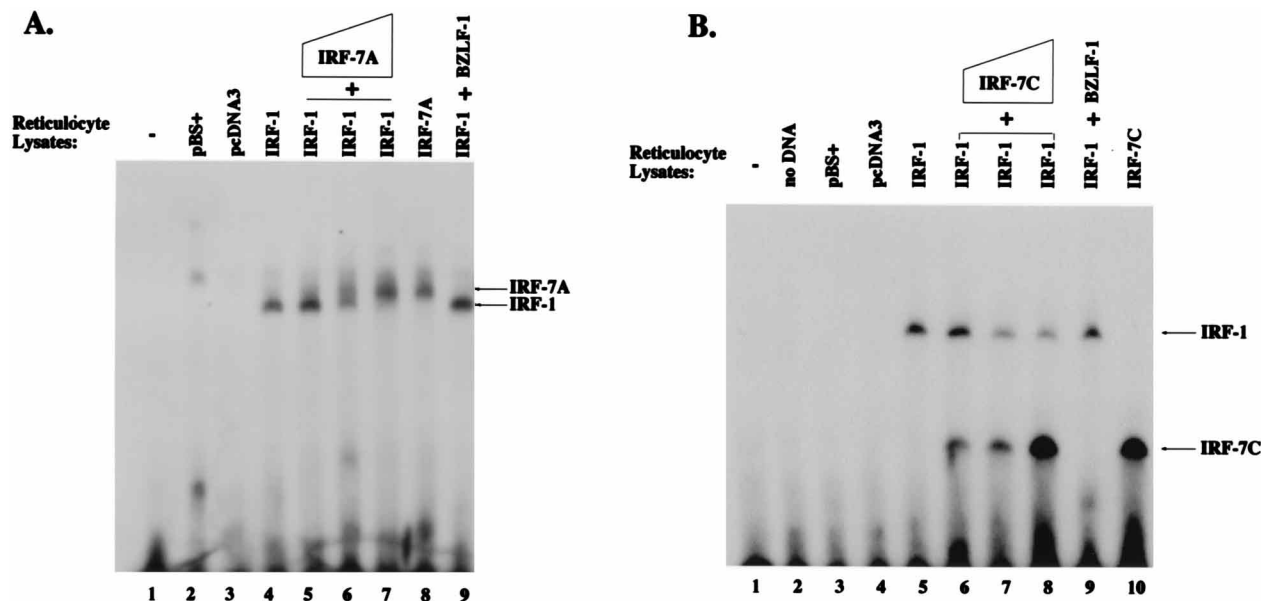


FIG. 9. IRF-7 can compete with IRF-1 for the consensus ISRE site. ³²P-labeled cellular ISG 15 ISRE probe and reticulocyte lysates containing in vitro-translated proteins from various plasmids were used for EMSA analysis. (A) In vitro-translated IRF-7A was used. Lanes: 1, free probe. 2 and 3, 8 μl of reticulocyte lysates translated from vector plasmid pBS+ (for IRF-1) and pcDNA3 (for IRF-7A), respectively; 4 to 7, 2 μl of lysates containing IRF-1 together with 0, 0.5, 2.5, and 6 μl of lysates containing IRF-7A, respectively (the total volume of lysates was made up to 8 μl with a lysate of pcDNA3); 8, 2 μl of lysates from pBS+ and 6 μl from IRF-7C; 9, 4 μl of lysates containing IRF-1 plus 6 μl of in vitro-translated EBV BZLF-1. (B) In vitro-translated IRF-7C was used. Lanes: 1, free probe; 2 to 4, 7 μl of reticulocyte lysates translated from no DNA, vector plasmid pBS+, and pcDNA3, respectively; 5 to 8, 4 μl of lysates containing IRF-1 together with 0, 0.5, 1.5, and 3 μl of lysates containing IRF-7C, respectively (the total volume of lysates was made up to 7 μl with a lysate of pcDNA3); 9, 4 μl of IRF-1 plus 3 μl of in vitro-translated EBV BZLF-1; 10, 4 μl of lysates from pBS+ and 3 μl from IRF-7C.

regulators suggest a complex network of the IFN-regulated genes. Further work is needed to reveal the functional significance of those common attributes. However, these factors may have distinctive cellular functions not necessary linked to IFN; e.g., Pip/LSIRF is able to interact with PU.1 and facilitate the binding of Pip/LSIRF to the enhancer of the immunoglobulin light-chain gene, converting it to a transcriptional activator (12).

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