MOLECULAR AND CELLULAR BIOLOGY, Feb. 1997, p. 873–886 Vol. 17, No. 2
0270-7306/97/$04.00+0

Constitutive Activation of Epstein-Barr Virus (EBV) Nuclear Antigen 1 Gene Transcription by IRF1 and IRF2 during Restricted EBV Latency

BRIAN C. SCHAEFER,[1]† EMILY PAULSON,[2] JACK L. STROMINGER,[1] AND SAMUEL H. SPECK[2]*

Division of Tumor Virology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115,[1] and Department of Pathology and Division of Molecular Oncology, Washington University School of Medicine, St. Louis, Missouri 63110[2]

Received 8 October 1996/Returned for modification 6 November 1996/Accepted 20 November 1996

The Epstein-Barr virus (EBV) EBNA1 gene promoter active in the type I program of restricted viral latency was recently identified and shown to reside in the viral *Bam*HI Q fragment. This promoter, Qp, is active in a wide variety of cell lines and has an architecture reminiscent of eukaryotic housekeeping gene promoters (B. C. Schaefer, J. L. Strominger, and S. H. Speck, Proc. Natl. Acad. Sci. USA 92:10565–10569, 1995; B. C. Schaefer, J. L. Strominger, and S. H. Speck, Mol. Cell. Biol. 17:364–377, 1997). Here we demonstrate by deletion analysis that the important *cis*-acting elements regulating Qp are clustered in a relatively small region (ca. 80 bp) surrounding the site of transcription initiation. Immediately upstream of the site of initiation is a region which is protected from DNase I digestion by crude nuclear extracts. Electrophoretic mobility shift analyses (EMSA) employing probes spanning this region demonstrated the presence of two major protein complexes. Deletion analysis of Qp demonstrated that at least one of these complexes plays an important role in Qp activity. Evidence that interferon response factor 2 (IRF2) is a major constituent of the most prominent EMSA complex and that IRF1 may be a minor component of this complex is presented. Transfections into IRF1$^{-/-}$, IRF2$^{-/-}$, and IRF1,2$^{-/-}$ fibroblasts demonstrated that absence of both IRF1 and IRF2 reduced Qp activity to approximately the same extent as mutation of the IRF-binding site in Qp, strongly implicating IRF2, and perhaps IRF1, in the regulation of Qp activity. Notably, transcription from Qp was not inducible by either alpha or gamma interferon in EBV-negative B cells but rather was shown to be constitutively activated by IRF1 and IRF2. This observation suggests that IRF1 and IRF2 have a previously unrecognized role as constitutive activators of specific genes. Additionally, data presented indicate that a protein complex containing the nonhistone architectural protein HMG-I(Y) binds to the region identified as the major transcription initiation site for Qp. This observation raises the possibility that HMG-I(Y)-induced DNA bending plays a role in the initiation of transcription from Qp.

Epstein-Barr virus (EBV) infection in immunocompetent humans is predominantly latent and persists for the life of the individual (reviewed in reference 41). Recently, it has been shown that EBV is capable of adopting at least three distinct forms of latency (27). Type III latency is observed upon in vitro infection of B lymphocytes and results in immortalization and continuous proliferation of the infected B cells via the action of a subset of the six EBV nuclear antigens (EBNAs; EBNA1, EBNA2, EBNA3a, EBNA3b, EBNA3c, and EBNA4) and three membrane proteins (LMP1, LMP2a, and LMP2b) expressed in the type III (immortalizing) program. However, in vivo, the type III latency program is likely to be only transiently observed upon initial infection of a naive host, since several of the type III latent antigens elicit a potent cytotoxic T-lymphocyte response resulting in very effective elimination of type III latently infected B cells (reviewed in reference 33).

EBV is also capable of entering programs of latency in which the expression of latent antigens is restricted with respect to the type III program. In type I latency, only the EBNA1 protein is expressed (64). It has recently been shown that EBNA1 peptides do not enter the major histocompatibility complex class I antigen processing and presentation pathway (29, 39, 52), and cells which express only EBNA1 are not detected by host cellular immune surveillance mechanisms (63). The type II latency program differs from type I latency only in the expression of variable combinations of LMP1, LMP2a, and LMP2b, in addition to EBNA1. Since the type I latency program was identified, there has been speculation that type I latently infected B lymphocytes represent the lifelong reservoir of virus in immunocompetent, seropositive individuals, and there have recently been several reports which provide evidence that a type I-like form of restricted viral latency does indeed exist in healthy carriers of EBV (6, 51, 60, 85).

Investigations into the molecular basis of type III and type I latency have demonstrated that distinct promoters are used to drive transcription of the EBNAs in each latent program. In type III latency, transcription of all six nuclear antigens is initiated from either Wp (during initial infection of resting B cells) or Cp (in cycling B cells), and the long primary transcripts are differentially spliced to generate the mature EBNA transcripts (75, 94; see Fig. 1A for a schematic representation of EBV latent transcription and locations of promoters). Recent investigations have shown that transcription of the EBNA1 gene in type I latency is driven by a promoter designated Qp, which is considerably downstream of the type III latency EBNA gene promoters (57, 70–72). Qp has an architecture with numerous similarities to eukaryotic housekeeping

* Corresponding author. Mailing address: Department of Pathology, Box 8118, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-0367. Fax: (314) 362-4096. E-mail: speck@pathology.wustl.edu.

† Present address: Howard Hughes Medical Institute, Denver, CO 80206-2761.

gene promoters (71, 73) and is silenced during type III latency by the binding of EBNA1 downstream of the transcription initiation site (68, 73; see Fig. 1B for the sequence and structural features of Qp).

Qp-driven EBNA1 gene transcription has been observed in a number of tumor cell lines of different cellular origins (73), and Qp is likely to initiate the EBNA1 transcripts which are observed in the absence of expression of other EBNA mRNAs in vivo (85). A number of studies have shown that the type III latency EBNA gene promoters, Cp and Wp, are inactivated by CpG methylation during type I latency (2, 10, 22, 44, 49, 62, 69), while Qp is at the center of a hypomethylated island and remains active (73). Although evidence strongly suggests that methylation of Cp and Wp is absolutely required for EBV to stably maintain the type I and II latency programs (73), almost nothing is known about the specific *trans*-acting factors which regulate transcription from Qp. We thus initiated an investigation based on our previous characterizations of Qp (71, 73) to identify the *trans*-acting proteins utilized by EBV to drive EBNA1 gene transcription during restricted viral latency.

MATERIALS AND METHODS

Cell lines and tissue culture. Akata (77), Mutu I (18), and Rael (31) are group I Burkitt's lymphoma (BL) cell lines. DG-75 (4), BL-41 (37), and Ramos (32) are EBV-negative BL-cell lines. BJAB is a non-Burkitt's B-cell lymphoma line (46, 100). The lymphoblastoid cell lines (LCLs) X50-7, JC5, and JY have been described and characterized previously (94, 95). The polyclonal LCLs AW, Boston, and Dana were established from thymic B lymphocytes (a gift from Ana Lena Spetz). K562 is a human chronic myelogenous leukemia cell line (42), and Jurkat is a human T-cell leukemia cell line (91). The above cell lines were propagated in RPMI 1640 supplemented with 10% fetal bovine serum. The HeLa cell line was derived from a human cervical carcinoma (information regarding the derivation of HeLa cells can be obtained from the American Type Culture Collection). CNE2 is an EBV-negative, adherent cell line derived from a nasopharyngeal biopsy (38). The NIH 3T3 murine fibroblast line (21) was obtained from the American Type Culture Collection, and IRF1$^{-/-}$, IRF2$^{-/-}$, and IRF1,2$^{-/-}$ murine embryonic fibroblasts (45) were used with the permission of Tadatsugu Taniguchi and were generously provided by Patricia Vaughan and Gary Stein. All adherent cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Generation of plasmids. Plasmids −1730FQUCAT, −122QUCAT (71, 73), and BGCAT (13) have been described previously. The −35QUCAT and −4QUCAT constructs were generated by *Sph*I-*Hin*dIII digestion of the FQUCAT construct, releasing a fragment containing all Q and U sequences downstream of +136, relative to Qp. PCR amplifications with sequence-specific primers having either an appended *Sac*I site (5′ end) or the natural *Sph*I site at +136 (3′ end) were used to generate the −35/+136 and −4/+136 fragments. These PCR fragments were digested with *Sac*I and *Sph*I and cloned with the *Sph*I-*Hin*dIII Q-U fragment (described above) into the *Sac*I-*Hin*dIII sites of the pGL2-CAT polylinker in a three-part ligation. Constructs having site-directed mutations in the Qp interferon (IFN) consensus sequence (ICS) (m-AT mutants) were generated by the *Taq* polymerase-*Taq* ligase three-primer PCR mutagenesis method of Michael (47) by employing the phosphorylated mutagenic primer 5′-CGCTTTGCGAAAAatAAAGTGCTTGAAA-3′. The FQULuc and FQULuc/m-AT plasmids were generated by replacing the chloramphenicol acetyltransferase (CAT) gene in the FQUCAT and FQUCAT/m-AT constructs with the luciferase gene. The pCMV-βGal (43) construct was a gift from Elizabeth Neuman and contains the β-galactosidase gene downstream of the immediate-early cytomegalovirus promoter.

Transfections, reporter gene assays, and cell cycle analyses. Transfection of nonadherent cells was done via electroporation as previously described (73). Adherent cells were transfected by lipofection as previously described (73), with the exception of the IRF2$^{-/-}$ cell line, which was transfected with 3.5 μg of plasmid. CAT assays were performed as previously described (17). In cell cycle analysis experiments, NIH 3T3 cells were transfected (in triplicate) by lipofection with both FQULuc (or FQULuc/m-AT; 8.5 μg) and pCMV-βGal (3 μg) on a single 100-mm-diameter dish. Following 14 h without serum (during incubation with the liposome-encapsulated DNA), medium containing 10% serum was added and cells were incubated at 37°C for 4 h. Cells were then trypsinized, replated in medium containing 10% serum at equal densities onto eight 100-mm dishes, and incubated for a further 4 h at 37°C. Cells were washed twice with phosphate-buffered saline (PBS), and medium containing 0.5% serum was added. Serum starvation was performed for 48 h at 37°C. Cells were then released from growth arrest by addition of medium containing 20% serum and harvested at the postrelease time points indicated in Results. Each sample (i.e., one 100-mm plate) was split into two equal volumes; the first portion was washed in PBS, fixed in ethanol, and stored at 4°C for cell cycle analysis by propidium iodide staining, and the second portion was used for luciferase and β-galactosidase assays. Luciferase assays and β-galactosidase assays (for normalization of luciferase values) were performed essentially as described by Krek et al. (34), and propidium iodide staining and cell cycle analysis were performed as described by Neuman et al. (56).

DNase I footprinting and electrophoretic mobility shift analyses (EMSA). The Qp DNase I footprinting construct was generated by cloning bases 1 to 249 (*Bam*HI to *Pvu*II) of the *Bam*HI Q fragment into the *Bam*HI and *Eco*RV sites of pBluescript-KS+ (Stratagene). Nuclear extracts were prepared and footprinting was performed as previously described (12).

For EMSA, crude nuclear extracts were prepared from ca. 10^8 cells by employing a modified version of the protocol described by Dignam et al. (8). Cells were pelleted by centrifugation, washed once with PBS, and resuspended in 4 volumes of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of antipain per ml, 0.5 mM NaF, 0.5 mM $NaVO_4$). Cells were incubated on ice for 1 h and lysed by 20 strokes with a Dounce homogenizer. Nuclei were separated from cytosolic components by being spun for 10 s in an Eppendorf Microfuge, and the supernatant was removed by aspiration. Nuclei were resuspended in 3 volumes of buffer B (10 mM HEPES [pH 7.9], 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of antipain per ml, 0.5 mM NaF, 0.5 mM $NaVO_4$) and incubated on ice for 30 min. Nuclei were then removed by centrifugation at 4°C for 20 min (14,000 × *g*), and supernatants were aliquoted and stored at −70°C.

Oligonucleotides were synthesized (AnaGen) and then purified on acrylamide denaturing gels (67) and chromatography on SepPak C_{18} columns (Waters). Complementary oligonucleotides were mixed at an equimolar ratio in 50 mM NaCl, heated to 90°C, and annealed by slow cooling. Labeling (100 ng) was achieved by a Klenow fill-in reaction (67) performed for 1 to 2 h at 4°C with [α-^{32}P]dGTP and [α-^{32}P]dCTP (New England Nuclear) and unlabeled dATP and dTTP in the labeling reaction. The labeled probe was separated from unincorporated nucleotides by chromatography over Sephadex G25 (Sigma).

For EMSA experiments, nuclear extracts were thawed on ice and 3 μl (5 to 10 μg) was mixed with 2 μl (20 μg) of purified bovine serum albumin (New England Biolabs), 0.4 μl (2 μg) of sheared salmon sperm DNA, 7 μl of buffer D (20 mM HEPES [pH 7.9], 0.1 M KCl, 20% glycerol, 0.2 mM EDTA, 1 mM DTT), and 11 μl of high-pressure liquid chromatography-grade water (per sample). An unlabeled oligonucleotide competitor (200 ng) was also added to the appropriate samples at this time. This mixture was incubated at room temperature for 20 min, the labeled oligonucleotide was added (1 ng), and the mixture was incubated for a further 20 min at room temperature. For supershift experiments, 2 μg of antibody [anti-IRF1 (C-20); mouse-specific anti-IRF1 (M-20), anti-IRF2 (C-19), or anti-ISGF3γ (C-20) (Santa Cruz Biotechnology); or polyclonal anti-human HMG-I(Y) (82) (a gift from Dimitris Thanos)] was then added, and samples were incubated at room temperature for an additional hour. Samples were loaded onto a 4% native acrylamide gel (40:1 acrylamide-bisacrylamide) and electrophoresed at 4°C at no higher than 45 mA (0.5× TBE buffer [1× TBE buffer is 90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3]). Gels were run for 2 to 5 h, transferred to Whatman paper, and dried. Retarded complexes were visualized by autoradiography (1 to 16 h at room temperature). For the oligonucleotides employed in EMSA related to the IRF studies, see Fig. 4A. The EBNA1 site oligonucleotides used (see Fig. 12A and B) are as follows (only the sense strand is shown): 2×EBNA1, 5′-CGCGCGGGATAGCGTGCGCTACCGGATGGCGGGTAATACATGCTATCCTTACCTAG-3′; upEBNA1, 5′-CTAGAAAAGGCGCGGGATAGCGTGCGCTGGATC-3′; dwnEBNA1, 5′-CTAGACATGCTATCCTTACATTTTGGATC-3′.

RESULTS

A region immediately upstream of Qp binds cellular factors and is required for reporter gene activity. To identify candidate *cis*-acting elements which might play a role in the regulation of Qp activity, crude nuclear extracts prepared from the group I BL-cell lines Akata and Rael (type I latency), the group III BL-cell line Jijoye (type III latency), and the EBV-negative BL-cell line DG75 were used in a DNase I protection analysis. All extracts protected a region extending approximately from −22 to −1 relative to the Qp transcription initiation site (Fig. 1B and 2). Since an identical protection pattern was observed with extracts prepared from both EBV-positive and EBV-negative cell lines, the factors binding to this region must be of cellular origin and not virally encoded. In addition, a distal region of Qp appeared to be protected from DNase I digestion by crude nuclear extract prepared from Rael cells (Fig. 2). This region corresponds to approximately −97 to

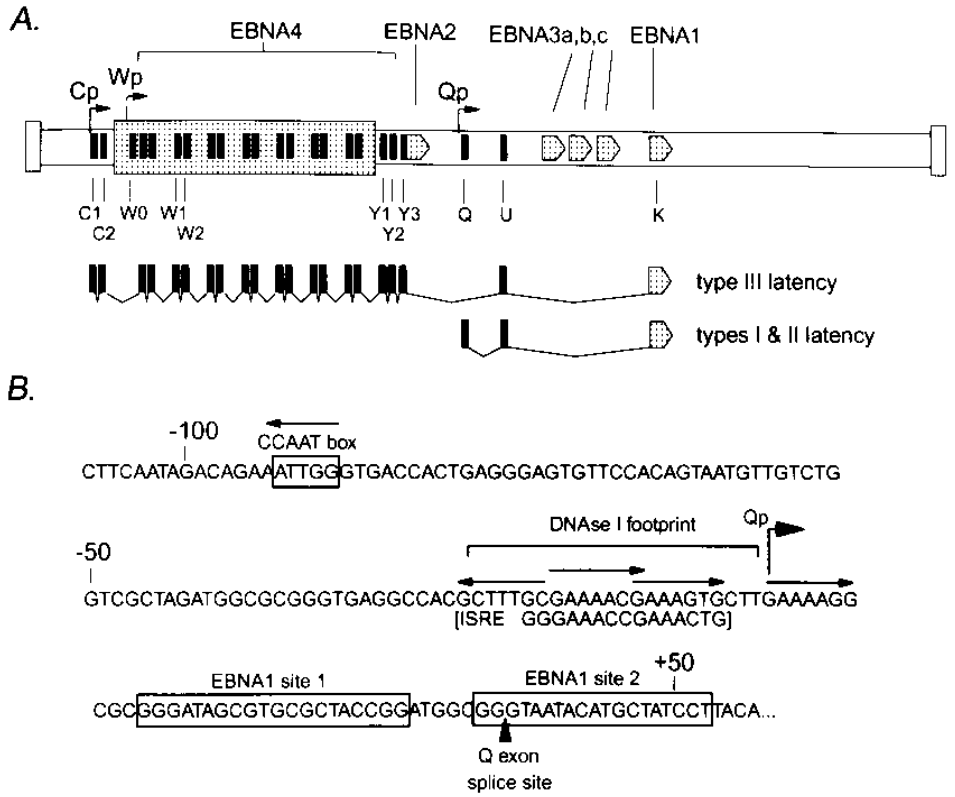

FIG. 1. (A) Simplified schematic representation of exon structures of EBNA1 gene transcripts expressed during either type III latency or type I or II latency. Regions encoding the various EBNA gene products are indicated on the viral genome. The large shaded rectangle depicts the major internal repeat (IR1) in EBV, which is composed of a variable number of 3-kb direct repeats. All EBNA transcripts expressed during type III latency have common 5′ exons (the W1 and W2 repeat exons encoded within IR1 and the Y1, Y2, and Y3 exons), which are alternatively spliced to downstream coding exons. EBNA gene transcription in type III latently infected B cells initiates from either Wp or Cp, while EBNA1 gene transcription in type I and II latently infected cells initiates from Qp. (B) Sequence of the Qp region of the EBV genome. The bent arrow denotes the major transcription initiation site identified by S1 nuclease protection (71). A region protected by crude nuclear extracts from DNase I digestion (see Fig. 2) is indicated by the bracket. Horizontal arrows within the DNase I footprint indicate a repeated sequence motif, and the homology to a characterized IFN-stimulated response element (ISRE) from the ISG15 promoter (40) is also shown. The low-affinity EBNA1-binding sites, the Q exon splice site, and an inverted CCAAT box are also indicated.

−127 relative to the Qp transcription initiation site. The significance of this protection is uncertain, since it was not observed with extracts prepared from the other cell lines.

To define the minimal sequences upstream of the Qp initiation site required for reporter construct activity, the previously described −1730FQUCAT construct (73; see the legend to Fig. 3) was subjected to deletion analysis. We have previously demonstrated by S1 nuclease protection that an equivalent reporter construct (FQUglobin, in which the CAT gene was replaced with the rabbit β-globin gene) specifically initiated transcription from Qp (71). Additionally, studies have shown that sequences downstream of the EBNA1-binding sites (i.e., beyond +75 relative to the Qp transcription initiation site) are not required for Qp activity (73, 73a). A series of reporter constructs containing truncations of upstream sequences was derived, and their activities were assessed by transient transfection into group I BL-cell lines (Fig. 3). This analysis demonstrated that reporter gene activity was significantly diminished when sequences between −35 and −4 bp relative to Qp were deleted but was relatively unaffected by deletion of sequences upstream of −35 bp. Thus, this deletion analysis demonstrated that a 31-bp region, which includes most of the cellular factor binding site identified by DNase I footprinting, contains a *cis* element(s) involved in upregulation of transcription initiation from Qp.

Identification of an ICS in Qp. Promoters which lack a TATAA element (such as Qp) are thought to direct RNA polymerase II to the site of transcription initiation primarily via an element called an initiator (Inr) at the transcription start site (reviewed in reference 90). Since the cellular factor binding site directly upstream of the Qp start site is important for Qp reporter activity, this factor may represent the Qp Inr element (QpInr). To further define the factors that bind to the putative QpInr element, a DNA fragment corresponding to the region protected from DNase I digestion was synthesized (Fig. 4A) and analyzed in an EMSA. Two major complexes were identified by using crude nuclear extract prepared from the group I BL-cell line Rael (Fig. 4B), as well as with nuclear extracts prepared from other group I BL-cell lines, LCLs, and EBV-negative BL-cell lines (data not shown). The faster-migrating (lower) complex (indicated by an arrow in Fig. 4B; note that this complex is actually composed of two distinct bands) and the slower-migrating (upper) complex were both competed for by an excess of the unlabeled QpInr oligonucleotide, demonstrating that binding is specific (Fig. 4B).

Inspection of the QpInr region revealed a strong homology to the consensus binding site for members of the IFN response factor (IRF) family (Fig. 4A). Indeed, the lower complex was competed for by a double-stranded oligonucleotide (ISRE) containing the ICS from a typical IFN-inducible gene, ISG15

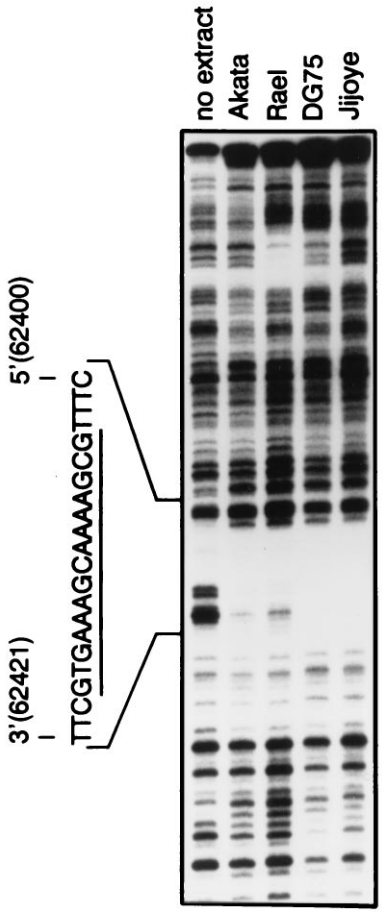

FIG. 2. DNase I footprinting analysis of the region upstream of Qp. Footprinting was carried out by employing crude nuclear extracts prepared from the indicated cell lines as described in Materials and Methods. The sequence of the core region protected from digestion is indicated to the left, and the ICS is underlined.

(40) (Fig. 4B). However, binding of the slower-migrating complex was not competed for by the ISRE oligonucleotide. Furthermore, competitor oligonucleotides containing a minimal mutation in the core of the ICS (m-AT) which eliminates a universally conserved G and abrogates binding of IRF family members (79) were unable to compete for the lower complex (Fig. 4B, QpInr m-AT and ISRE m-AT). Finally, a smaller oligonucleotide (QpInr-IRF) which contains only the QpInr putative IRF site was able to compete for the lower complex but was unable to compete for the upper complex. The latter result, in conjunction with the failure of the ISRE oligonucleotide to compete for the upper complex, indicates that the slower-migrating (upper) complex requires sequences other than (or in addition to) those required for IRF binding.

To further investigate cellular factor binding to the QpInr putative ICS, EMSA were carried out by employing crude nuclear extracts from a panel of cell lines (Fig. 5) and either the QpInr-IRF probe or the ISG15 ISRE probe (Fig. 4A contains the probe sequences). The patterns of complex formation obtained with the QpInr-IRF probe were very similar with all of the nuclear extracts employed (Fig. 5), suggesting that the cellular factors involved are ubiquitous. However, with the ISRE probe, the EMSA pattern obtained with extracts prepared from B cells was distinctly different from that observed with extracts prepared from either the T-cell line Jurkat or the epithelial cell line HeLa or CNE2. Based on the studies of Bovolenta et al. (5), the additional complex observed with the B-cell extracts and the ISRE probe is likely to reflect binding of the IRF family member ICSBP, which is expressed only in lymphoid tissues (but is also known not to be expressed in Jurkat cells) (5, 55). ICSBP heterodimerizes with IRF1 and IRF2, and such heterodimers are reported to bind to ICS sites and inhibit promoter activity in unstimulated cells (5, 92). Notably, this complex does not appear to form with the QpInr ICS; rather, the complex formed appears to be identical to the complex observed with extract from nonlymphoid cell lines and

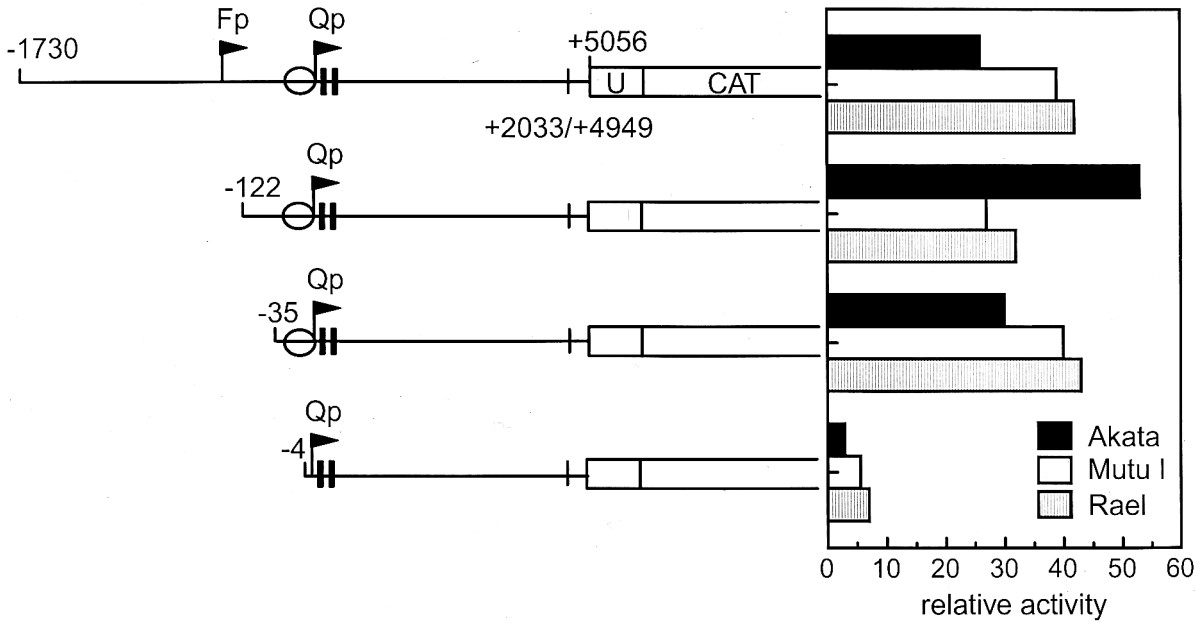

FIG. 3. Activities of Qp reporter constructs demonstrating that sequences upstream of −35 bp are not required for efficient transcript initiation. The indicated reporter constructs were transfected in the group I BL-cell lines Akata, Mutu I, and Rael as described in Materials and Methods. CAT activities are given relative to the activity of a CAT reporter construct driven by a minimal promoter composed of the β-globin gene TATA box. An upstream promoter, Fp, which is active during the early stages of lytic replication (70) is also shown.

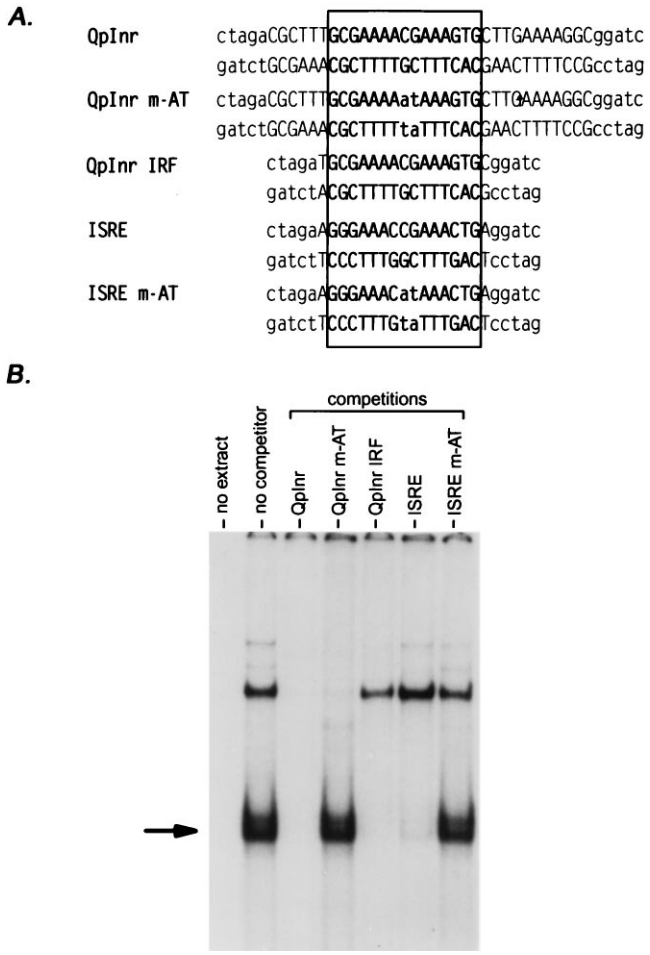

FIG. 4. Competition for binding to the Qp putative ICS element by the ISRE probe but not by a mutant ISRE which does not bind IRF family members. (A) Sequences of oligonucleotide probes employed to assess binding to the QpInr region. The putative ICS element present in Qp is highlighted in boldface within the boxed region and is aligned with the ICS element in the ISRE probe. The sequences of the probes containing a minimal mutation in the core of the Qp and ISRE ICS elements (m-AT mutants) are also shown. (B) EMSA of a cellular factor binding to the QpInr region. Crude nuclear extract prepared from the Rael BL-cell line was employed with the ^{32}P-labeled QpInr probe as described in Materials and Methods. Competitions were carried out by addition of 200 ng of the indicated unlabeled oligonucleotide probes. An arrow indicates the complex which, based on the mutation and competition analyses shown, binds to the Qp ICS element.

the ISRE probe (Fig. 5), suggesting that the complex bound to the QpInr-IRF oligonucleotide does not contain ICSBP.

IRF1 and IRF2, but not ISGF3γ, are bound to the Qp ICS. To determine whether known members of the IRF family might be involved in binding to the Qp ICS, specific antibody reagents were employed in EMSA. When cell extracts prepared from the Rael group I BL-cell line were used in conjunction with the QpInr-IRF oligonucleotide probe, an antibody which specifically recognizes IRF2 (Santa Cruz Biotech) was able to supershift the faster-migrating complex almost completely (note that with the QpInr-IRF oligonucleotide probe, the slower-migrating complex observed with the QpInr oligonucleotide did not form) (Fig. 6). An antibody which specifically recognizes IRF1 (Santa Cruz Biotech) was able to generate a weak supershift, suggesting that a small fraction of this complex reflects IRF1 binding (data not shown). However, when an antibody which recognizes the IRF family member

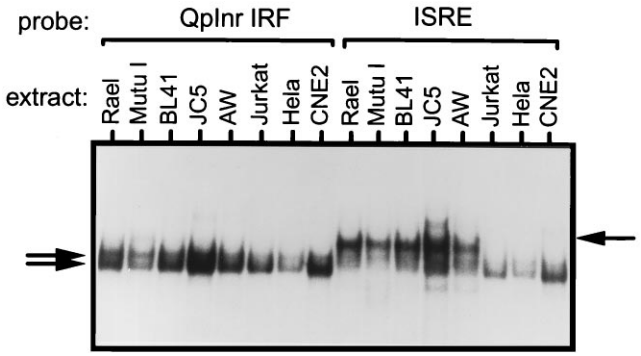

FIG. 5. Comparison of cellular factor binding to the Qp ICS and the ISRE sites. Crude nuclear extracts from the indicated cell lines were prepared as described in Materials and Methods. Rael, Mutu I, BL41, JC5, and AW are B-cell lines, while Jurkat is a T-cell line and HeLa and CNE2 are epithelial in origin. The two arrows to the left indicate the faster-migrating complex shown in Fig. 4B (which is shown in Fig. 6 to reflect IRF2 binding). The arrow to the right indicates a slightly slower-migrating complex with characteristics consistent with binding of IRF1-ICSBP and/or IRF2-ICSBP heterodimers (see the text for a discussion).

ISGF3γ (p48 subunit of the ISGF3 transcription factor [28, 89]; Santa Cruz Biotech) was employed, no supershift was detected (Fig. 6), indicating that this factor is most likely not involved in binding to this site. A very faint complex observed below the major ISRE-bound complex (but not below the complex bound by the QpInr-IRF oligonucleotide; compare the JC5 lanes in Fig. 5) was reproducibly supershifted by the anti-ISGF3γ antibody, demonstrating that this antibody is

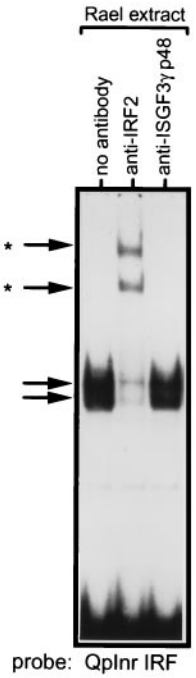

FIG. 6. Evidence that the Qp ICS element binds predominantly IRF2. A supershift EMSA employing antibodies specific for either IRF2 or ISGF3γp48 was carried out as described in Materials and Methods. The arrows with asterisks indicate the positions of the supershifted complexes observed upon addition of anti-IRF2 antibody. A weak supershift was observed with anti-IRF1 antibody (data not shown). The sequence of the QpInr IRF probe used is shown in Fig. 4A.

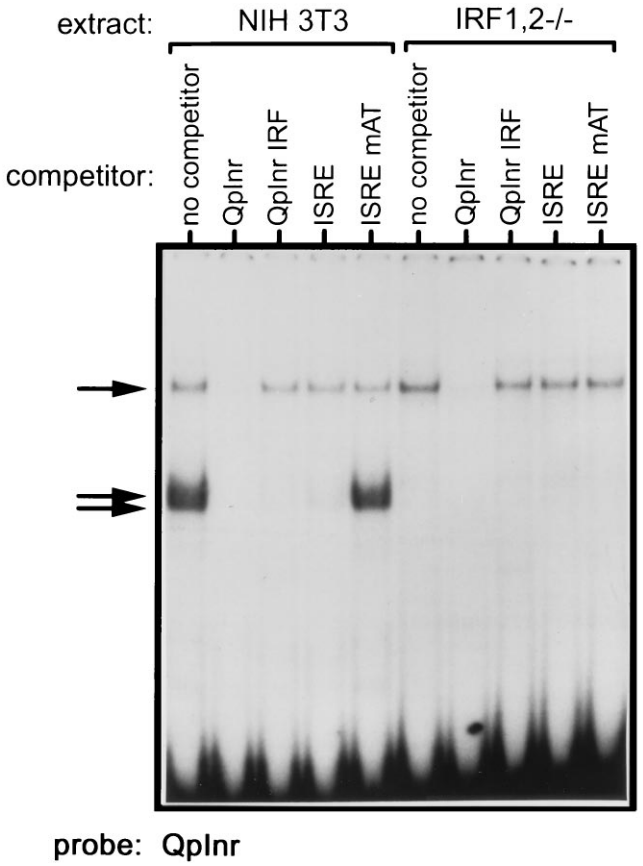

FIG. 7. Failure to form a complex with the Qp ICS element due to lack of IRF1 and IRF2. Cellular factor binding to the QpInr region was analyzed by employing nuclear extract prepared from mouse fibroblast cell lines containing (NIH 3T3) or lacking (IRF1,2$^{-/-}$) IRF1 and IRF2. The two closely spaced arrows to the left denote the doublet corresponding to binding of IRF1 and IRF2, while the upper arrow denotes the slower-migrating complex which is not influenced by mutation of the Qp ICS element. Competitions were carried out as described in Materials and Methods. The sequences of the probe and competitor oligonucleotides used are shown in Fig. 4A.

functional and specific (data not shown). Others have also observed that unstimulated cells contain the ISGF3γ protein, which binds to the ISG15 ISRE oligonucleotide (28) and migrates faster than IRF1 and IRF2 homodimers (5).

To further examine IRF1 and IRF2 binding to the Qp ICS, extracts were prepared from fibroblast cell lines established from mice lacking either IRF1 (IRF1$^{-/-}$), IRF2 (IRF2$^{-/-}$), or both IRF1 and IRF2 (IRF1,2$^{-/-}$) (45). Initially, the EMSA pattern obtained with a cell extract prepared from wild-type NIH 3T3 cells was assessed by employing the QpInr probe (Fig. 7). The pattern observed was indistinguishable from the pattern observed with extracts prepared from human cell lines (compare Fig. 7 with Fig. 4B). As previously observed with group 1 BL extracts (see above), the QpInr-IRF oligonucleotide and the ISRE oligonucleotide competitors competed for the faster-migrating (lower) complex but not the upper complex. As expected, the ISRE m-AT oligonucleotide competitor did not compete for either complex. An extract prepared from the double-knockout cell line (IRF1,2$^{-/-}$) was able to form only the upper complex (Fig. 7), substantiating the antibody supershift evidence that the faster-migrating complex is composed of IRF2 and IRF1 binding. These observations also indicate that the formation of the upper complex does not involve either IRF1 or IRF2, consistent with the failure of antibodies which recognize these factors to supershift this complex (data not shown).

When an extract prepared from the IRF1$^{-/-}$ cell line was used in conjunction with the QpInr-IRF probe, the observed banding pattern of the faster-migrating complex was indistinguishable from that observed with an extract from unmutated cells (Fig. 8A). A similar pattern was also observed when the labeled ISRE probe was employed, although an additional, slower-migrating band was also observed (Fig. 8A). As shown above, the faster-migrating complex was competed for by self and the ISRE competitor oligonucleotide probe but not by the ISRE m-AT mutant probe (Fig. 8A). Finally, antibodies against IRF2 supershifted the QpInr-IRF-bound complex nearly completely, while antibodies which specifically recognize IRF1 or ISGF3γ were unable to supershift this complex. When an extract prepared from the IRF2$^{-/-}$ cell line was employed in conjunction with the QpInr-IRF probe, a weak complex was observed which migrated with the leading edge of the faster-migrating complex (which, as indicated above, appears to be composed of two distinct bands). This complex was competed for by self and the ISRE but not by the ISRE m-AT mutant, and it was supershifted only by the anti-IRF1 antibody (Fig. 8A and B). This result suggests that IRF1 binding is a small proportion of the faster-migrating complex in wild-type cells, although it is also possible that IRF1 binding might occur only in the absence of IRF2. The striking similarity of the IRF2-containing complexes formed with an extract prepared from IRF1$^{-/-}$ cells to those formed with an extract from IRF1-containing cells appears to support the latter contention and also raises the possibility that heterodimers composed of IRF2 and another IRF family member are involved in the regulation of Qp. These results also demonstrate the specificity of the antibody reagents employed in the EMSA and provide strong support for the identification of IRF2 as the major cellular factor in unstimulated cell binding to the Qp ICS.

Mutation of the Qp ICS has a variable effect on Qp activity. To assess the impact of mutation of the Qp IRF-binding site on promoter activity, Qp reporter constructs were generated in which the m-AT mutation (which was shown above to inhibit IRF binding; for specific mutations, refer to Fig. 4A) was introduced into the Qp IRF-binding site. Both the unmutated and m-AT mutant Qp reporter constructs were transiently transfected into a panel of EBV-positive and EBV-negative cell lines (Fig. 9A). In all cases, the m-AT mutation significantly diminished activity, although the magnitude of this effect varied between cell lines. In the EBV-negative HeLa and DG75 cell lines, Qp activity was diminished 15- to 20-fold. The effect of the m-AT mutation in the group I BL-cell lines Mutu I and Rael was more modest, giving rise to only a five- to sixfold reduction in activity. It should be noted that in the latter cases the m-AT mutant exhibited significant activity, indicating that IRF binding is not essential for Qp activity in some cell lines.

To determine whether the identification of IRF binding by EMSA correlated with Qp activity in vivo, the wild-type and m-AT mutant Qp reporter constructs were introduced into mouse fibroblast cell lines lacking either IRF1, IRF2, or both IRF1 and IRF2 (45) (Fig. 9B). When these reporter constructs were transfected into the wild-type (NIH 3T3) cell line, the effect of the m-AT mutation was of a magnitude similar to that observed in the group I BL-cell lines. The IRF1$^{-/-}$ cell line gave results very similar to those obtained with the wild-type NIH 3T3 cell line, while the impact of the m-AT mutation was slightly diminished in IRF2$^{-/-}$ cells. Importantly, when both IRF1 and IRF2 were knocked out, the activities of the wild-

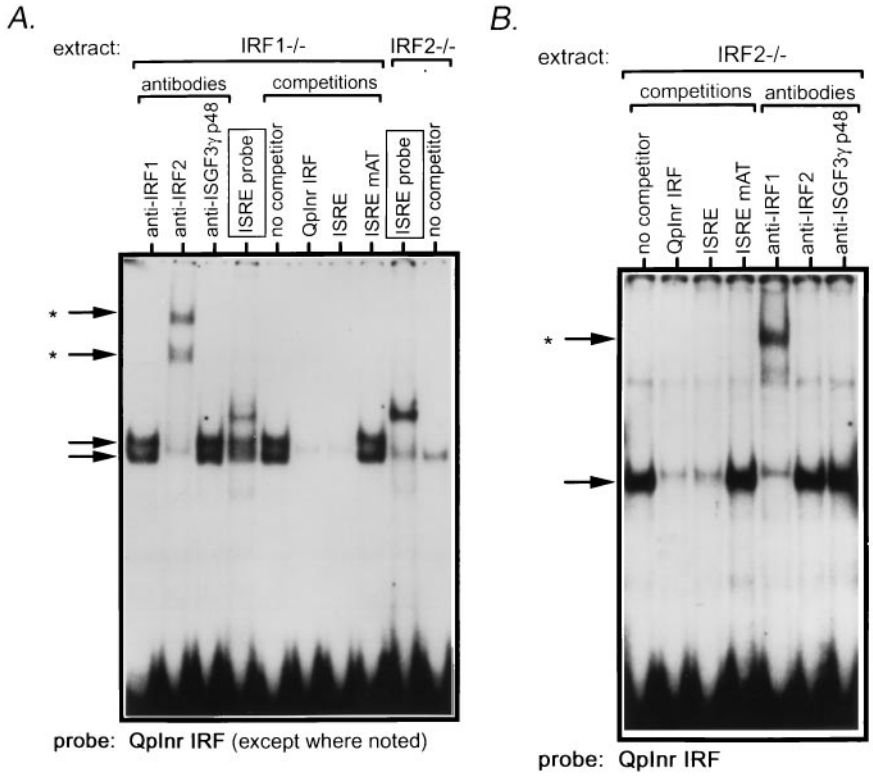

FIG. 8. Demonstration that both IRF1 and IRF2 bind to the Qp ICS element employing nuclear extracts from cell lines lacking either IRF1 or IRF2. (A) EMSA employing the QpInr IRF probe, except where it is indicated that the ISRE probe was employed, with nuclear extract prepared from mouse fibroblast cell lines lacking either IRF1 (IRF1$^{-/-}$) or IRF2 (IRF2$^{-/-}$). (B) EMSA of cellular factor binding to the QpInr IRF probe employing crude nuclear extract prepared from a mouse fibroblast cell line lacking IRF2 (IRF2$^{-/-}$). This exposure is darker than that in panel A (compare no-competitor lane with IRF2$^{-/-}$ extract in A and B). Competitions and antibody supershifts were carried out as described in Materials and Methods. Arrows with asterisks show the positions of supershifted complexes. Sequences of the probe and competitor oligonucleotides used are shown in Fig. 4A.

type and m-AT mutant Qp reporter constructs were indistinguishable. Thus, these results confirm the role of IRF1 and IRF2 in the activation of Qp transcription.

Qp is not IFN inducible in established B-cell lines. Since many promoters that contain an ICS are IFN inducible (40), the EBV-positive B-cell lines Rael, Akata, and JY were treated with either IFN-α, IFN-γ, or both to determine if transcription from the endogenous viral Qp could be upregulated by either IFN. Cells were mock treated or treated with 300 U of IFN-α, IFN-γ, or IFN-α plus IFN-γ per ml for 0, 4, 12, 24, 48 or 78 h, and then total RNA was isolated from the treated cells. The relative levels of Qp-initiated transcripts were assessed by reverse transcription-PCR (72). No differences between IFN-treated and untreated cells were detected (data not shown), indicating that Qp is not inducible by either IFN-α or IFN-γ. To further substantiate these findings, DG-75 cells (EBV-negative BL cells) were transfected with either an IFN-inducible IRF1 promoter construct (IRF1pCAT), the −1730FQUCAT construct, or the −1730FQUCAT/m-AT construct (which contains the same m-AT mutation in the Qp ICS described above). Following transfection, the cells were treated with IFN-γ or mock treated. Neither the −1730FQUCAT nor the −1730FQUCAT/m-AT construct demonstrated increased CAT activity following IFN-γ treatment (data not shown). In contrast, the IRF1pCAT construct showed a modest (about sixfold) induction of CAT activity following IFN-γ treatment of the transfected DG-75 cells. Thus, these experiments confirm that the Qp ICS does not confer IFN inducibility upon Qp, consistent with the observation that the Qp ICS does not appear to bind ISGF3γ in EMSA (ISGF3γ is the primary DNA-binding component of the transcription factor ISGF3, which is the principal mediator of the response to IFN-α [28, 40, 58]). Interestingly, the higher levels of IRF1 induced by IFN-γ do not result in upregulation of Qp activity, even though

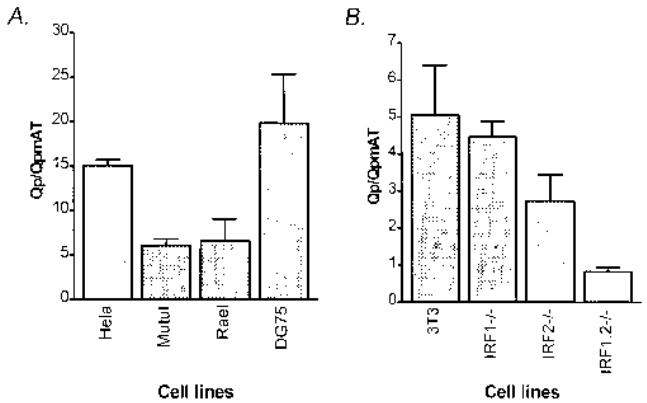

FIG. 9. Activation of transcription from Qp by both IRF1 and IRF2. (A) Comparison of the activities of unmutated and ICS m-AT mutated −1730FQUCAT reporter constructs. The structure of the −1730FQUCAT reporter construct is shown in Fig. 3. The position of the m-AT mutation introduced into the Qp ICS element is shown in Fig. 4A. The ratio of the observed activities with the unmutated and mutated reporter constructs in the indicated cell lines is shown. (B) Ratio of observed activities of the unmutated and m-AT mutated reporter constructs in mouse fibroblast cell lines containing (NIH 3T3) or lacking IRF1 (IRF1$^{-/-}$), IRF2 (IRF2$^{-/-}$), or both IRF1 and IRF2 (IRF1,2$^{-/-}$). Transfections and reporter assays were carried out as described in Materials and Methods.

Element	Sequence	interferon responsive
ISRE	AGGGAAACCGAAACTGA	+
QpInrIRF	TGCGAAAACGAAAGTGC	-
Histone H4	TTGAAAACCGAAAGCGC	-
PRDI	GGGAGAAGTGAAAGTGG	-
IFNα1	ACAGAAATGGAAAGTGG	-
VCAM1	AGTGAAATAGAAAGTCT	-
TAP1(ICS1)	AGCGAAATCAGGGGCGG	-
$H2K^b$ IRS	TGCAGAAGTGAAACTGT	+
Factor B	AAGGAAACAGAAACTGC	+
2'-5'OAS	TGAGGAAACGAAACCAA	+
6 16	AGGGAAAATGAAACTGC	+

FIG. 10. Alignment of known ICS elements and their responsiveness to induction by IFN-α/β (ISRE [40], histone H4 [88], PRDI [89], IFN-α1 [14], VCAM1 [74], TAP1 [48], $H2K^b$ IRS [30], factor B [96], 2′-5′ OAS [66], and 6-16 [59]). The nucleotide at position 14, which appears to correlate with IFN-α/β inducibility, is boxed.

IRF1 can clearly bind to the Qp ICS (Fig. 8B) and can upregulate Qp activity (in the absence of IRF2; Fig. 9B). This observation suggests that either (i) Qp is maximally active in uninduced cells and thus is not responsive to increased levels of IRF1 protein, (ii) IRF2 has a substantially higher affinity than IRF1 for the Qp ICS and increased levels of IRF1 therefore do not significantly influence transcription from Qp, or (iii) most of the additional IRF1 produced following IFN treatment is sequestered in complexes which do not bind with high affinity to the Qp ICS (such as IRF1-ICSBP heterodimers; Fig. 5). Further experiments are required to distinguish between these possibilities.

Since Qp does not appear to be IFN inducible and the Qp ICS does not bind ISGF3γ in vitro (see above), the Qp ICS element was compared with the sequence of a well-characterized ICS element from the IFN-inducible gene ISG15 (Fig. 10; designated ISRE and QpInrIRF, respectively) to identify nucleotide differences which might be responsible for these atypical properties of the Qp ICS. This comparison revealed that there are five nucleotide differences between ISRE and QpInrIRF, at positions 1, 3, 8, 14, and 17. In a previous study, the in vitro binding properties of the ICS element from the IFN-inducible gene ISG15 (ISRE) and the ICS element from the IFN-β gene (PRDI) were compared, revealing that whereas ISRE can bind either IRF1 or ISGF3γ with high affinity, PRDI binds only IRF1. For each of the six positions (of the 15 core nucleotides) at which the two ICSs differed, a mutant ISRE oligonucleotide was generated in which the corresponding PRDI nucleotide was substituted at that position and the binding properties of these mutant oligonucleotides were analyzed. One of these substitutions, corresponding to the boxed nucleotide (position 14) in Fig. 10, was found both to reduce the affinity of ISRE for ISGF3γ and to increase its affinity for IRF1 (89). Interestingly, QpInrIRF shares this same C-to-G substitution with the PRDI element, and this is the only position where both the ISRE and PRDI sequences differ and the Qp position matches that of the PRDI element.

Since the ICS elements of Qp and the IFN-β promoters share certain properties (neither bind ISGF3γ in vitro or confer IFN inducibility upon its promoter [see above and references 26 and 89]), the sequences of other ICS elements were examined. This sequence analysis (Fig. 10) revealed that most, if not all, of the characterized IFN-inducible promoters have a C at position 14 (ISRE, reference 40; $H2K^b$ IRS, reference 30; factor B, reference 96; 2′-5′ OAS, reference 66; 6-16, reference 59), which is consistent with the previously described consensus binding site for ISGF3γ, GGRAAAR(A/T)GAAACAR (40). In contrast, the promoters having an ICS element with a G at position 14 (Fig. 10) have generally been characterized as non-IFN-α/β inducible, with the ICS playing the role of a constitutive activator of transcription. Qp (see above), the IFN-β promoter (26), and all characterized IFN-α promoters (14) are not inducible by IFNs and have a G at position 14 of the ICS element which mediates constitutive activation of the promoter. It should also be noted that although the IFN-α and IFN-β promoters are both induced by virus, this induction has been shown to be mediated by factors other than IRF1 and IRF2 (14, 83). Additional examples of constitutive ICS elements are provided by the TAP1 and VCAM1 promoters. Although the VCAM1 (74) and TAP1 (48) promoters are inducible by IFN-γ, this induction is not mediated through the ICS elements and detailed analyses of these promoters have shown that one function of the ICSs of these two promoters is constitutive positive modulation of transcription. Finally, the histone H4 promoters of the FO108 gene (88) and of the other known human, mouse, and rat histone H4 genes (86) contain ICS elements with a G at position 14. The FO108 ICS element has been studied in considerable detail, and IRF1 and IRF2 have been shown to activate transcription of this histone H4 gene (88) and may also cooperate with proteins which confer cell cycle regulation on this promoter (87; see below). To our knowledge, the histone H4 promoters have not been shown to be IFN inducible. Thus, there appear to be a number of promoters which contain ICS elements that have a G (versus a C) at position 14 and are not responsive to IFN-α/β stimulation, presumably due to a reduced affinity for ISGF3γ (see above and reference 89). Such promoters are positively regulated by binding of IRF1 (and, in some cases, IRF2 [88]) to these variant ICS elements (Fig. 10).

Transcription from Qp is constitutive. To date, the only other promoter for which positive regulation by both IRF1 and IRF2 has been demonstrated is the histone H4 promoter FO108 (88). Transcription from the FO108 promoter is cell cycle regulated, with peak transcription occurring during S phase. The FO108 ICS is found in the cell cycle element of this promoter, and the ICS overlaps with a binding site for a distinct complex containing CDC2, cyclin A, and a member of the RB family (87). Similar to the histone H4 genes, the only known requirement for EBNA1 in EBV-infected cells involves the binding of EBNA1 to the EBV latent origin of replication (*oriP*) during S phase of the cell cycle, thereby allowing the EBV episome to be replicated coordinately with cellular genomic DNA (98, 99). Thus, EBNA1 might be produced in a cell cycle-regulated manner, with peak production occurring

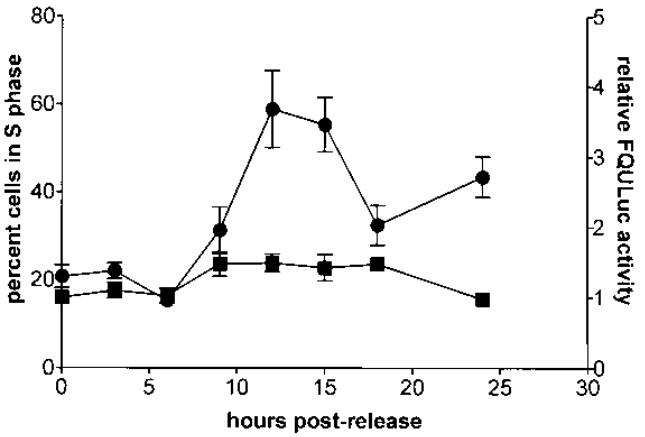

FIG. 11. Evidence that Qp is not cell cycle regulated. The −1730FQULuc and pCMV-βGal reporter plasmids were transfected into NIH 3T3 cells, and the cell cycle was arrested by serum starvation for 48 h as described in Materials and Methods. Following addition of 20% serum, aliquots of transfected cells were harvested at the indicated time points and analyzed for DNA content by propidium iodine staining, as well as β-galactosidase and luciferase activities. β-Galactosidase activity was used to normalize for variability in transfection efficiency and sample handling. Symbols: ■, relative Qp activity; ●, cells in S phase.

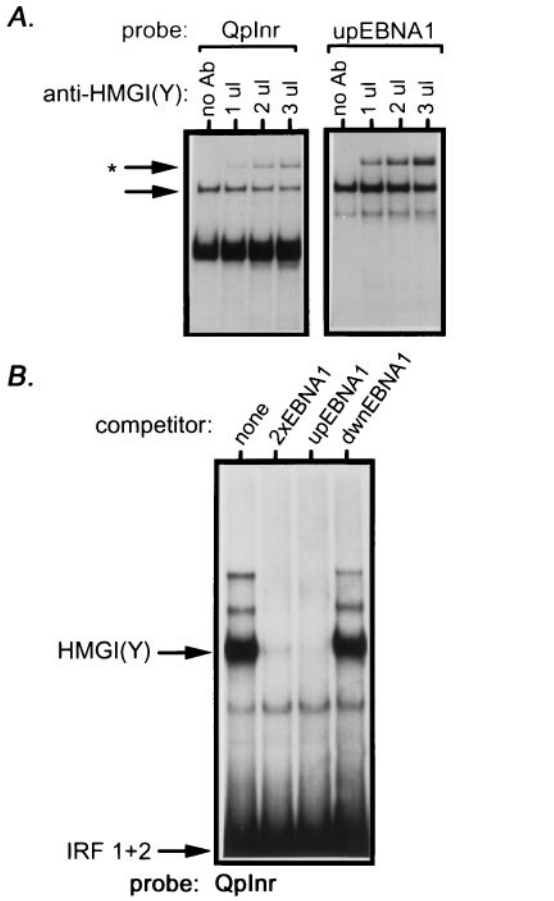

FIG. 12. Presence of the architectural protein HMG-I(Y) in a complex which binds near the Qp transcription initiation site. (A) Supershift EMSA of HMG-I(Y) binding to either the QpInr or upstream EBNA1 site (upEBNA1) oligonucleotide probe. The sequence of the QpInr probe is given in Fig. 4A, and the sequence of the upEBNA1 probe is given in Materials and Methods. Antibody (Ab) supershift EMSA were carried out as described in Materials and Methods by employing a polyclonal antibody generously provided by D. Thanos. (B) Competition by the upstream EBNA1 site oligonucleotide, but not the downstream EBNA1 site oligonucleotide, for HMG-I(Y) binding to the QpInr probe. Binding competition assays were carried out with 200 ng of the indicated oligonucleotide competitor as described in Materials and Methods. The sequences of the competitor oligonucleotides are given in Materials and Methods.

during S phase, analogous to the regulation of the histone H4 gene. Additionally, a previous study (76) reported an undefined E2F complex binding to a region of Qp overlapping the EBNA1-binding sites. E2F is a heterodimeric complex consisting of an E2F protein and a DP protein, which acts on a number of promoters to enable cell cycle-regulated transcription with peak activity occurring in late G_1-early S phase (reviewed in reference 35). Those investigators postulated that E2F binding might result in cell cycle regulation of Qp, although no data testing this hypothesis was presented.

To determine whether Qp activity is cell cycle regulated or constitutive, the −1730FQULuc and −1730FQULuc/m-AT constructs (identical to the −1730FQUCAT and −1730FQUCAT/m-AT constructs, respectively, except that the CAT gene has been replaced with the luciferase gene; the luciferase protein has a much shorter half-life than the CAT protein and is thus preferred for cell cycle analyses) were transfected into NIH 3T3 cells. These cells were cotransfected with pCMV-βGal so that the activities observed at various time points could be normalized to an internal standard. The transfected cells were then serum starved to synchronize the cells at G_0 and then released from growth arrest via addition of serum. The NIH 3T3 transfectants were harvested at eight time points ranging from 0 to 24 h postrelease. Cells were split into two fractions; the first fraction was used in a luciferase assay and a β-galactosidase assay to determine normalized Qp activity, and the second aliquot was stained with propidium iodide to determine the cell cycle distribution. As shown in Fig. 11, −1730FQULuc activity remained essentially unchanged over the time course, even though the number of cells in S phase ranged from approximately 18% at $t = 6$ h to 60% at $t = 12$ h. Analogous results were obtained with the −1730FQULuc/m-AT construct (data not shown). The above evidence thus indicates that Qp activity is not cell cycle regulated.

Additionally, we have been unable to confirm the results of Sung et al. (76). Although the region of the Qp EBNA1 sites identified as an E2F-binding site by Sung et al. (76) is capable of binding in vitro-translated GST-E2F-1, it is not clear that the cellular factor from Jurkat cell extracts which they observed in EMSA contained E2F. Indeed, we have detected a relatively weak protein complex which has the properties reported by Sung et al. (76); specifically, the complex binds in the region which represents approximately the middle third of the Qp EBNA1-binding site region, and this complex is competed for by an oligonucleotide encoding the E2F-binding site from the c-*myc* promoter but is not competed for by oligonucleotides encoding the E2F sites from the E2F-1 promoter or the dihydrofolate reductase promoter (76; data not shown). However, in contrast to the weak supershift of this Qp-bound protein complex with an anti-E2F-1 antibody reported by Sung et al. (76), we observed no supershift with an anti DP-1 antibody (or with an antibody directed against E2F-1), although the E2F complexes bound to an oligonucleotide encoding the dihydrofolate reductase promoter E2F-binding site were completely supershifted by this same anti-DP-1 antibody (data not shown). Based on the above data, it seems highly unlikely that an E2F complex binds in the Qp EBNA1 site region. All evidence thus supports the conclusion that transcription from Qp is consti-

tutive, consistent with its housekeeping promoter architecture (71, 73).

The cellular architectural protein HMG-I(Y) binds to the QpInr. The above analyses provide substantial evidence that transcription from Qp is upregulated by the binding of IRF2 and IRF1 to an ICS immediately upstream of the site of transcription initiation (Fig. 1B shows its location). However, EMSA identified two complexes binding to the QpInr oligonucleotide (Fig. 4A shows the sequence), and oligonucleotide competition and gel supershift analyses indicated that IRF1 and IRF2 are components of only the faster-migrating lower complexes but not the slower-migrating upper complex. The EMSA also demonstrated that a shorter oligonucleotide, QpInr-IRF (Fig. 4A), bound only the IRF2-IRF1 complex and not the upper complex (Fig. 4B and 5). Thus, the additional bases 3′ of the ICS (5′-TTGAAAAGGC-3′) appear to be necessary for binding of the upper complex to the QpInr oligonucleotide. Since Qp has certain functional characteristics in common with the IFN-β promoter, namely, positive regulation by a non-IFN-inducible ICS, the binding sequence preferences of transcription factors known to bind to the minimal IFN-β promoter were examined to identify candidates which might bind to 5′-TTGAAAAGGC-3′. This analysis identified the high-mobility group protein HMG-I(Y), which binds to the sequence 5′-GGGAAATTCC-3′ in the IFN-β promoter (82). Although this sequence is not strikingly similar to the Qp sequence shown above, HMG-I(Y) is known to have a rather loose specificity for A-T-rich sequences (61), which the motifs in Qp and the IFN-β promoter do have in common. EMSA were thus performed with the QpInr oligonucleotide, Rael crude nuclear extracts, and various amounts of a polyclonal anti-human HMG-I(Y) antibody (kindly supplied by D. Thanos). The anti-HMG-I(Y) antibody partially supershifted the upper (non-IRF1/2) complex, and the amount of material supershifted increased with the amount of antibody added (Fig. 12A). Additionally, we had previously observed that a complex which binds to the 5′ end of the Qp upstream EBNA1-binding site (upEBNA1 oligonucleotide) comigrated with the QpInr upper complex. This comigrating upEBNA1-bound complex was also supershifted in a dose-dependent manner by the anti-HMG-I(Y) antibody (Fig. 12A).

Since the experiments in Fig. 12A suggested that the comigrating complexes bound to the QpInr and upEBNA1 oligonucleotides both contain HMG-I(Y), competition analyses were performed to provide evidence that these two complexes have identical (or overlapping sets of) constituents. Crude Rael cell nuclear extracts were used in an EMSA with the labeled QpInr probe, and competitions were performed with either the intact Qp EBNA1 sites (2×EBNA1 oligonucleotide), the 5′ third of the Qp EBNA1 site region (upEBNA1 oligonucleotide), or the 3′ third of the Qp EBNA1 site region (dwnEBNA1 oligonucleotide). This competition demonstrated that both the 2×EBNA1 and the upEBNA1 oligonucleotides competed specifically for the HMG-I(Y)-containing complex bound to the QpInr (but not the IRF1-IRF2 complex), whereas the dwnEBNA1 oligonucleotide did not compete for this complex (Fig. 12B). It is important to note that although the sequence of the upEBNA1 oligonucleotide overlaps partially the QpInr oligonucleotide (bases GAAAAGGC), the 2×EBNA1 oligonucleotide does not include any overlapping sequence. The protein complex(es) containing HMG-I(Y) appears to bind to two distinct regions: the Qp sequences included in the 3′ end of the QpInr oligonucleotide and the sequences at the 5′ end of the 2×EBNA1 oligonucleotide. Further investigation is required to determine whether there exist two distinct binding sites for HMG-I(Y), or whether binding of the HMG-I(Y)-containing complex to sequences downstream of the Qp transcription initiation site is mediated by a separate DNA-binding activity with which HMG-I(Y) associates (see Discussion).

DISCUSSION

In this study, regulation of the type I latency EBNA1 promoter, Qp, was examined. Transient transfection of group I BL-cell lines employing a series of reporter constructs having nested 5′ deletions demonstrated that sequences upstream of −35 bp have little impact on reporter gene activity, while sequences between −35 and −4 bp significantly upregulated Qp activity. A sequence which overlaps the region defined as important for enhancement of Qp activity was protected from DNase I cleavage (−22 to −1 bp) in an in vitro footprinting assay employing nuclear extracts from EBV-positive and -negative BL-cell lines. EMSA using crude nuclear extracts also demonstrated specific in vitro binding of two predominant protein complexes to an oligonucleotide spanning the region protected from DNase I digestion. Further analyses were performed with specific antibody reagents, oligonucleotide competitors, and nuclear extracts from human cell lines and fibroblast lines derived from IRF1$^{-/-}$, IRF2$^{-/-}$, and IRF1,2$^{-/-}$ mice. These studies demonstrated that the faster-migrating EMSA complex represents primarily binding of IRF2, with IRF1 representing a small portion of the complex.

Several lines of evidence indicate that the binding of IRF2 and IRF1 observed in vitro is responsible for the upregulation of Qp by the −35 to −4 region identified by deletion analysis. First, the same mutation (m-AT) which abolishes the binding of IRF1 and IRF2 in vitro results in a reduction in Qp activity to approximately the same extent as that caused by deletion of the sequences between −35 and −4 bp. Also, analysis of reporter construct activity in IRF1 and IRF2 mutant cell lines demonstrated that cell lines having either IRF1 or IRF2 (or both factors) generated higher levels of transcription from the wild-type Qp construct than from the construct having the m-AT ICS mutation. However, in cells lacking expression of either IRF1 or IRF2, no difference in reporter gene activity was observed between wild-type and m-AT mutant Qp constructs.

Analysis of the ICS region from Qp, as well as from a number of other promoters, strongly suggests the existence of two distinct classes of ICS elements which can be functionally distinguished by the presence of either a C or a G at position 14 (Fig. 10). The C-14 variants are responsive to induction by IFN-α/β due to their high affinity for ISGF3γ (28, 40, 58), which is the primary DNA-binding component of ISGF3, a multisubunit transcription factor which mediates the response to IFN-α/β (see below). In contrast, this report and previously published data (89) suggest that the G-14 variants have a reduced affinity for ISGF3γ but bind IRF1 and IRF2 with high affinity. The latter ICS elements are not IFN responsive and are capable of constitutively activating transcription (14, 26, 48, 74).

EMSA also strongly suggest that the predominant lymphocyte-derived complex bound to the ISG15 ISRE probe is an IRF2-ICSBP (and/or an IRF1-ICSBP) heterodimer, whereas the Qp ICS appears to have a much lower affinity for the ICSBP heterodimeric complexes. Since ICSBP-containing heterodimers inhibit transcription (5, 54), it seems likely that promoters of IFN-stimulated genes, such as ISG15, are repressed in unstimulated B cells (55) by the binding of the ICSBP-IRF2 and ICSBP-IRF1 heterodimers. Indeed, ICSBP is capable of repressing multiple IFN-responsive promoters

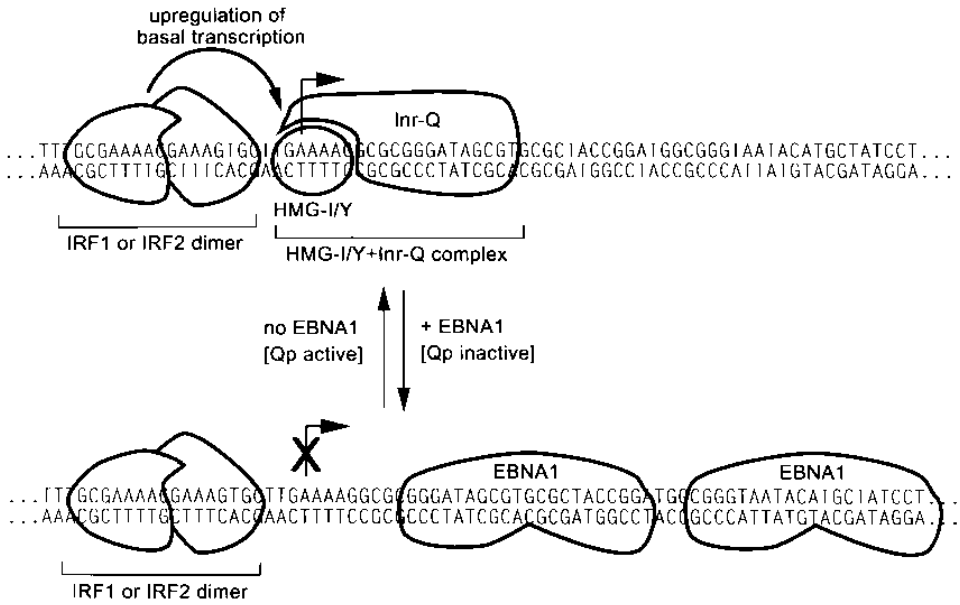

FIG. 13. Model for activation of Qp by IRF1 and IRF2 and repression by EBNA1. The presence of an Inr complex composed of HMG-I(Y) and Inr-Q is hypothetical, and the nucleotide requirements for Inr-Q binding have not been determined (see Discussion).

(54, 92) and this repression is overcome by treatment of cells with IFNs (92). In contrast, ICS elements which provide constitutive activation, such as the Qp ICS, are likely to bind IRF2 and IRF1 homodimers preferentially rather than the inhibitory ICSBP-containing heterodimers. It is compelling to speculate that all C-14 ICS elements (IFN stimulated) bind ICSBP-IRF1 and ICSBP-IRF2 heterodimers with higher affinity than IRF1 and IRF2 homodimers, whereas the opposite is true of the G-14 ICS elements (constitutive activators). However, this hypothesis has not been rigorously tested.

IRF1 was originally cloned from a cDNA expression library based on its affinity for a synthetic oligonucleotide $(AAGTGA)_4$. A single hexamer of this sequence and several similar purine-rich hexamers were found in the virus-inducible region of the IFN-β promoter, and IRF1 was believed to be responsible for both virus-induced transcription of the IFN-β gene and upregulation of transcription of genes induced by IFN-α/β (50). However, subsequent research has revealed that IRF1 is a member of a large family of transcription factors which bind to similar to identical sequences. Evidence indicates that most IFN-induced genes are activated primarily by the multisubunit gene ISGF3 (28, 40, 58) and that NF-κB is the principal mediator of virus induction of the IFN-β gene (58, 83). Indeed, targeted disruption of the IRF1 gene did not result in diminished production of IFN-α/β in response to virus infection, and the induction of several IFN-induced genes was also normal (65). The $IRF1^{-/-}$ mouse did show certain unexpected defects, including substantially decreased production of $CD8^+$ thymocytes (45). Additionally, transcription of inducible nitric oxide synthase by macrophages in response to stimulation with IFN-γ and lipopolysaccharide is critically dependent upon IRF1 (25), as is production of interleukin-1β converting enzyme by thymocytes in response to concanavalin A and γ irradiation (78). Multiple lines of evidence thus indicate that the principal functions of IRF1 are not related to virus-stimulated or IFN-mediated responses.

As in the case of IRF1, the principal role of IRF2 seems to have little to do with its initially ascribed function. IRF2 was cloned on the basis of sequence homology with the IRF1 gene, and functional studies suggested that IRF2 can act as a repressor of IRF1-mediated activation of the IFN-β gene, as well as other promoters which could be induced by IRF1 (19). Although IRF2 knockout mice indeed demonstrated moderately (two- to threefold) increased peak levels of IFNα/β in response to viral infection, the kinetics of induction were identical (45). In fact, postinduction repression of the IFN-β gene has been shown to be mediated by a non-IRF family protein, PRDI-BF1, which binds to the IFN-β PRDI element (26), and perhaps also by the binding of homodimers of the rel family protein p50 to the PRDII element (83). Constitutive repression of the IFN-β gene is believed to be mediated by the binding of an unidentified factor to a region which overlaps the PRDII domain (but not the PRDI-ICS site) (15, 16). IRF2 is thus not responsible for the repression of transcription from the IFN genes, and it does not contribute to the postinduction decrease in IFN gene transcription. However, the $IRF2^{-/-}$ mice did demonstrate a generalized hematopoietic defect in which the most readily observed phenotype was a reduction in the number of mature B and T lymphocytes in the bone marrow (45). The observations that overexpression of IRF2 leads to oncogenic transformation (20) and that IRF1 is a tumor suppressor (80) which can overcome IRF2-mediated transformation (20) underscores the above evidence that IRF1 and IRF2 play major roles in the regulation of diverse processes relating to cell growth. Analysis of the available evidence thus argues that a major role (or perhaps even the primary role) of IRF1 and IRF2 is the constitutive activation of a number of different promoters and that the absence of this transactivation in IRF1 and IRF2 knockout mice may account for the observed defects in processes related to growth and development (reviewed in reference 81).

Recently, it was reported that both IRF1 and IRF2 contribute to the positive regulation of the histone H4 gene FO108 (88). A latent activation domain of IRF2 had previously been characterized by investigators who speculated that IRF2 could act as a positive transcriptional regulator under certain circumstances (97). Qp provides a second example of a promoter that is positively regulated by both IRF1 and IRF2. Interestingly,

both of these promoters drive transcription of genes which are intimately involved in DNA replication (production of the histone H4 gene FO108 product is cell cycle regulated and coupled to DNA replication; the EBNA1 protein is required for latent replication of the EBV genome), perhaps suggesting that IRF1 and IRF2 play a generalized role in the regulation of eukaryotic DNA replication.

EMSA results presented in Fig. 12A provide evidence that HMG-I(Y) is a constituent of the other major complex which binds in the positive regulatory domain of Qp. Qp is thus similar to the IFN-β and VCAM-1 promoters, which have binding sites for HMG-I(Y) and IRF1-IRF2 (53, 82, 84). As shown in Fig. 10, all three of these promoters have an ICS which is not IFN responsive and has a G at position 14, rather than the C which is found in IFN-inducible ICS elements. It thus seems that the IRF1 and IRF2 complexes which bind to the ICS(G-14) often cooperate with HMG-I(Y) to positively regulate transcription.

We speculate that, as illustrated in Fig. 13, the complex containing HMG-I(Y) binds directly downstream of IRF2 and IRF1, in a region overlapping the Qp transcription initiation sites. HMG-I(Y) is most likely to bind directly to the sequence TTGAAAA, since this is the only A-T-rich region in the QpInr oligonucleotide [which binds the HMG-I(Y)-containing complex] which is not in the QpInr-IRF oligonucleotide [which does not bind the HMG-I(Y)-containing complex]. Since the same HMG-I(Y)-containing complex also appears to bind to the 2×EBNA1 oligonucleotide (which does not contain the TTGAAAA sequence of the QpInr oligonucleotide), we postulate that there is an independent binding site for the HMG-I(Y)-containing complex. Because this binding site appears to be in the 5′ third of the 2×EBNA1 oligonucleotide, which has only the trinucleotide ATA as a potential HMG-I(Y) binding site, a non-HMG-I(Y) member of the complex is likely to provide the DNA-binding activity in this region (Fig. 13). A number of proteins have been shown to associate with HMG-I(Y), including NF-κB p50 (82, 84, 93), Oct-6 (36), Oct-2A (1), Elf-1 (23), and ATF-2 (9, 93). Current investigations are directed towards determining whether these and/or other proteins are associated with HMG-I(Y) in these Qp EMSA complexes.

The mechanism by which EBNA1 downregulates transcription from Qp has not been elucidated. Evidence indicates that each EBNA1 site contributes equally to repression, that the summed effects of the two sites are additive (not synergistic), and that the mechanism does not involve a simple blockade of polymerase progression by EBNA1 (73). One possibility, illustrated in Fig. 13, is that the binding of EBNA1 to Qp physically displaces the HMG-I(Y)-containing complex, thereby preventing transcription initiation or greatly diminishing its efficiency. However, since the complex containing HMG-I(Y) appears to have DNA contacts in the region of the upstream Qp EBNA1 binding site, but not the downstream EBNA1 binding site, a more elaborate mechanism may be required to explain the observation that each EBNA1 site mediates repression of Qp with equivalent efficiency.

It has recently been shown that HMG-I(Y) is a DNA bending protein (11, 61) and that positive regulation of transcription by HMG-I(Y) is dependent upon this bending property (11). Investigation of the mechanism by which the binding of EBNA1 to the EBV latency origin of replication (*oriP*) makes possible the replication of the EBV episome has revealed that EBNA1 is also a DNA bending protein. Two EBNA1 dimers bound to properly spaced adjacent sites interact to effect a major bend in the DNA helix and by a distinct mechanism, a single EBNA1 dimer bound to DNA induces a less substantial bend (reviewed in reference 7). Given that the spacing of the Qp EBNA1 sites is such that the two dimers would be on opposite sides of the DNA helix (3, 24), only the second mechanism is likely to be operative. Repression may thus be a result of the EBNA1-induced DNA bending distorting the bend induced by HMG-I(Y) in such a way that transcription is no longer facilitated by HMG-I(Y). Perhaps it is more likely, since HMG-I(Y) has been shown to bind to the minor grove of DNA with little sequence specificity but high specificity for specific conformations of DNA (61), that repression by EBNA1-induced bending lowers the affinity of HMG-I(Y) for the Qp regulatory region, thereby facilitating the "competition" illustrated in the model shown in Fig. 13 via local distortion of the DNA helix.

ACKNOWLEDGMENTS

We thank T. Taniguchi for permission to use the IRF knockout fibroblast cell lines and P. Vaughan and G. Stein for supplying frozen cultures of the IRF knockout cell lines and for advice regarding their propagation. We also thank D. Thanos for providing the anti-human HMG-I(Y) polyclonal antibody, Robert Schreiber for the IRF1 promoter-driven CAT reporter construct and human IFN-α and IFN-γ, Elizabeth Neuman for technical advice concerning cell cycle analyses and for providing the pCMV-βGal plasmid, and Erik Flemington for critical discussions concerning data presented in this report.

REFERENCES

1. **Abdulkadir, S. A., S. Krishna, D. Thanos, T. Maniatis, J. L. Strominger, and S. J. Ono.** 1995. Functional roles of the transcription factor Oct-2A and the high mobility group protein I/Y in HLA-DR gene expression. J. Exp. Med. **182:**487–500.
2. **Altiok, E., J. Minarovits, H. Li-Fu, B. Contreras-Brodin, G. Klein, and I. Ernberg.** 1992. Host-cell-phenotype-dependent control of BCR2/BWR1 promoter complex regulates the expression of Epstein-Barr virus nuclear antigens 2–6. Proc. Natl. Acad. Sci. USA **89:**905–909.
3. **Ambinder, R. F., W. A. Shah, D. R. Rawlins, G. S. Hayward, and S. D. Hayward.** 1990. Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in the Epstein-Barr virus DNA. J. Virol. **64:**2369–2379.
4. **Ben-Bassat, H., N. Goldblum, S. Mitrani, T. Goldblum, J. M. Yoffey, M. M. Cohen, Z. Bentwitch, B. Ramot, E. Klein, and G. Klein.** 1977. Establishment in culture of a new type of lymphocyte from a "Burkitt-like" lymphoma (line D.G.-75). Int. J. Cancer **19:**27–33.
5. **Bovolenta, C., P. H. Driggers, M. S. Marks, J. A. Medin, A. D. Politis, S. N. Vogel, D. E. Levy, K. Sakaguchi, E. Appella, J. E. Coligan, and K. Ozato.** 1994. Molecular interactions between interferon consensus sequence binding protein and members of the interferon regulatory factor family. Proc. Natl. Acad. Sci. USA **91:**5046–5050.
6. **Chen, F., J.-Z. Zou, L. di Renzo, G. Winberg, L.-F. Hu, E. Klein, G. Klein, and I. Ernberg.** 1995. A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP-1. J. Virol. **69:**3752–3758.
7. **Dean, F. B., and M. O'Donnell.** 1996. DNA-protein interactions: two steps to binding replication origins? Curr. Biol. **6:**931–934.
8. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
9. **Du, W., and T. Maniatis.** 1994. The high mobility group protein HMG I(Y) can stimulate or inhibit DNA binding of distinct transcription factor ATF-2 isoforms. Proc. Natl. Acad. Sci. USA **91:**11318–11322.
10. **Ernberg, I., K. Falk, J. Minarovits, P. Busson, T. Tursz, M. G. Masucci, and G. Klein.** 1989. The role of methylation in the phenotype-dependent modulation of Epstein-Barr nuclear antigen 2 and latent membrane protein genes in cells latently infected with Epstein-Barr virus. J. Gen. Virol. **70:**2989–3002.
11. **Falvo, J. V., D. Thanos, and T. Maniatis.** 1995. Reversal of intrinsic DNA bends in the IFNβ gene enhancer by transcription factors and the architectural protein HMG I(Y). Cell **83:**1101–1111.
12. **Flemington, E., and S. H. Speck.** 1990. Identification of phorbol ester responsive elements in the promoter of Epstein-Barr virus putative lytic switch gene BZLF1. J. Virol. **64:**1217–1226.
13. **Flemington, E. K., J. P. Lytle, C. Cayrol, A. Borras, and S. H. Speck.** 1994. DNA-binding-defective mutants of the Epstein-Barr virus lytic switch activator Zta transactivate with altered specificities. Mol. Cell. Biol. **14:**3041–3052.
14. **Genin, P., J. Braganca, N. Darracq, J. Doly, and A. Civas.** 1995. A novel

PRDI and TG binding activity involved in virus-induced transcription of IFN-α genes. Nucleic Acids Res. **23:**5055–5063.
15. **Goodbourn, S., H. Burstein, and T. Maniatis.** 1986. The human β-interferon gene enhancer is under negative control. Cell **45:**601–610.
16. **Goodbourn, S., and T. Maniatis.** 1988. Overlapping positive and negative regulatory domains of the human β-interferon gene. Proc. Natl. Acad. Sci. USA **85:**1447–1451.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
18. **Gregory, C. D., M. Rowe, and A. B. Rickinson.** 1990. Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. J. Gen. Virol. **71:**1481–1495.
19. **Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi.** 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell **58:**729–739.
20. **Harada, H., M. Kitagawa, N. Tanaka, H. Yamamoto, K. Harada, M. Ishihara, and T. Taniguchi.** 1993. Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. Science **259:**971–974.
21. **Jainchill, J. L., S. A. Aaronson, and G. J. Todaro.** 1969. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J. Virol. **4:**549–553.
22. **Jansson, A., M. Masucci, and L. Rymo.** 1992. Methylation of discrete sites within the enhancer region regulates the activity of the Epstein-Barr virus *Bam*HI W promoter in Burkitt lymphoma lines. J. Virol. **66:**62–69.
23. **John, S., R. B. Reeves, J.-X. Lin, R. Child, J. M. Leiden, C. B. Thompson, and W. J. Leonard.** 1995. Regulation of cell-type-specific interleukin-2 receptor α-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-κB family proteins. Mol. Cell. Biol. **15:**1786–1796.
24. **Jones, C. H., S. D. Hayward, and D. R. Rawlins.** 1989. Interaction of the lymphocyte-derived Epstein-Barr virus nuclear antigen EBNA-1 with its DNA-binding sites. J. Virol. **63:**101–110.
25. **Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S. I. Koh, T. Kimura, S. L. Green, T. W. Mak, T. Taniguchi, and J. Vilcek.** 1994. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science **263:**1612–1615.
26. **Keller, A. D., and T. Maniatis.** 1991. Identification and characterization of a novel repressor of β-interferon gene expression. Genes Dev. **5:**868–879.
27. **Kerr, B. M., A. L. Lear, M. Rowe, D. Croom-Carter, L. S. Young, S. M. Rookes, P. H. Gallimore, and A. B. Rickinson.** 1992. Three transcriptionally distinct forms of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage. Virology **187:**189–201.
28. **Kessler, D. S., S. A. Veals, X.-Y. Fu, and D. E. Levy.** 1990. Interferon-α regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. Genes Dev. **4:**1753–1765.
29. **Khanna, R., S. R. Burrows, M. G. Kurilla, C. A. Jacob, I. S. Misko, T. B. Sculley, E. Kieff, and D. J. Moss.** 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J. Exp. Med. **176:**169–176.
30. **Kimura, A., A. Israel, O. Le Bail, and P. Kourilsky.** 1986. Detailed analysis of the mouse H2-K^b promoter: enhancer-like sequences and their role in the regulation of class I gene expression. Cell **44:**261–272.
31. **Klein, G., L. Dombos, and M. Gothosokar.** 1972. Sensitivity of Epstein-Barr virus (EBV) producer and non-producer human lymphoblastoid cell lines to superinfection with EB-virus. Int. J. Cancer **10:**44–57.
32. **Klein, G., B. Giovanella, A. Westman, J. S. Stehlin, and D. Mumford.** 1975. An EBV-genome-negative cell line established from an American Burkitt lymphoma; receptor characteristics. EBV infectability and permanent conversion into EBV-positive sublines by *in vitro* infection. Intervirology **5:**319–334.
33. **Klein, G.** 1994. Epstein-Barr virus strategy in normal and neoplastic B cells. Cell **77:**791–793.
34. **Krek, W., D. M. Livingston, and S. Shirodokar.** 1993. Binding to DNA and retinoblastoma gene product promoted by complex formation of different E2F family members. Science **262:**1557–1560.
35. **Lam, E. W., and N. B. La Thangue.** 1994. DP and E2F proteins: coordinating transcription with cell cycle progression. Curr. Opin. Cell Biol. **6:**859–866.
36. **Leger, H., E. Sock, K. Renner, F. Grummt, and M. Wegner.** 1995. Functional interaction between the POU domain protein Tst-1/Oct-6 and the high-mobility-group protein HMG-I/Y. Mol. Cell. Biol. **15:**3738–3747.
37. **Lenoir, G. M., M. Vuillaume, and C. Bonnardel.** 1985. The use of lymphomatous and lymphoblastoid cell lines in the study of Burkitt's lymphoma. IARC Sci. Publ. **60:**309–318.
38. **Lerman, M. I., A. Sakai, K. T. Yao, and N. H. Colburn.** 1987. DNA sequences in human nasopharyngeal carcinoma cells that specify susceptibility to tumor promoter-induced neoplastic transformation. Carcinogenesis **8:**121–127.
39. **Levitskaya, J., M. Coram, V. Levitsky, S. Imreh, P. M. Steigerwald-Mullen, G. Klein, M. G. Kurilla, and M. G. Masucci.** 1995. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. Nature **375:**685–688.
40. **Levy, D. E., D. S. Kessler, R. Pine, N. Reich, and J. E. Darnell.** 1988. Interferon-induced nuclear factors that bind to a shared promoter element correlate with positive and negative transcriptional control. Genes Dev. **2:**383–393.
41. **Liebowitz, D., and E. Kieff.** 1993. Epstein-Barr virus, p. 107–172. *In* B. Roizman, R. J. Whitley, and C. Lopez (ed.), The human herpes viruses. Raven Press, New York, N.Y.
42. **Lozzio, C. B., and B. B. Lozzio.** 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood **45:**321–334.
43. **MacGregor, G. R., and C. T. Caskey.** 1989. Construction of plasmids that express *E. coli* beta-galactosidase in mammalian cells. Nucleic Acids Res. **17:**2365.
44. **Masucci, M. G., B. Contreras-Salazar, E. Ragnar, K. Falk, J. Minarovits, I. Ernberg, and G. Klein.** 1989. 5-Azacytidine up regulates the expression of Epstein-Barr virus nuclear antigen 2 (EBNA-2) through EBNA-6 and latent membrane protein in the Burkitt's lymphoma line Rael. J. Virol. **63:**3135–3141.
45. **Matsuyama, T., T. Kimura, M. Kitagawa, K. Pfeffer, T. Kawakimi, M. Watanabe, T. M. Kundig, R. Amakawa, K. Kishihara, A. Wakeham, J. Potter, C. L. Furlonger, A. Narendran, H. Suzuki, P. S. Ohashi, C. J. Paige, T. Taniguchi, and T. W. Mak.** 1993. Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. Cell **75:**83–97.
46. **Menezes, J., W. Liebold, G. Klein, and G. Clements.** 1975. Establishment and characterization of an Epstein-Barr virus (EBV)-negative lymphoblastoid B cell line (B-JAB) from an exceptional EBV-genome negative African Burkitt's lymphoma. Biomedicine **22:**276–284.
47. **Michael, S. F.** 1994. Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. BioTechniques **16:**410–412.
48. **Min, W., J. S. Pober, and D. R. Johnson.** 1996. Kinetically coordinated induction of TAP1 by IFN-γ: the rapid induction of TAP1 by IFN-γ is mediated by STAT1α. J. Immunol. **156:**3174–3183.
49. **Minarovits, J., L.-F. Hu, S. Minarovits-Kormuta, G. Klein, and I. Ernberg.** 1994. Sequence-specific methylation inhibits the activity of the Epstein-Barr virus LMP1 and BCR2 enhancer-promoter regions. Virology **200:**661–667.
50. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi.** 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. Cell **54:**903–913.
51. **Miyashita, E. M., B. Yang, K. M. C. Lam, D. H. Crawford, and D. A. Thorley-Lawson.** 1995. A novel form of Epstein-Barr virus latency in normal B cells *in vivo*. Cell **80:**593–601.
52. **Murray, R. J., M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff, and A. B. Rickinson.** 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. J. Exp. Med. **176:**157–168.
53. **Neish, A. S., M. A. Read, D. Thanos, R. Pine, T. Maniatis, and T. Collins.** 1995. Endothelial interferon regulatory factor 1 cooperates with NF-κB as a transcriptional activator of vascular cell adhesion molecule 1. Mol. Cell. Biol. **15:**2558–2569.
54. **Nelson, N., M. S. Marks, P. H. Driggers, and K. Ozato.** 1993. Interferon consensus sequence-binding protein, a member of the interferon regulatory factor family, suppresses interferon-induced gene transcription. Mol. Cell. Biol. **13:**588–599.
55. **Nelson, N., Y. Kanno, C. Hong, C. Contursi, T. Fujita, B. J. Fowlkes, E. O'Connell, J. Hu-Li, W. E. Paul, D. Jankovic, A. F. Sher, J. E. Coligan, A. Thornton, E. Appella, Y. Yang, and K. Ozato.** 1996. Expression of IFN regulatory factor family proteins in lymphocytes: induction of Stat-1 and IFN consensus sequence binding protein expression by T cell activation. J. Immunol. **156:**3711–3720.
56. **Neuman, E., E. K. Flemington, W. R. Sellers, and W. G. Kaelin, Jr.** 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. **14:**6607–6615.
57. **Nonkwelo, C., J. Skinner, A. Bell, A. Rickinson, and J. Sample.** 1996. Transcription start sites downstream of the Epstein-Barr virus (EBV) Fp promoter in early-passage Burkitt lymphoma cells define a fourth promoter for expression of the EBV EBNA-1 protein. J. Virol. **70:**623–627.
58. **Pine, R., T. Decker, D. S. Kessler, D. E. Levy, and J. E. Darnell, Jr.** 1990. Purification and cloning of interferon-stimulated gene factor 2 (ISGF2): ISGF2 (IRF-1) can bind to the promoters of both beta interferon- and interferon-stimulated genes but is not a primary transcriptional activator of either. Mol. Cell. Biol. **10:**2448–2457.
59. **Porter, A. C. G., Y. Chernajovsky, T. C. Dale, C. S. Gilbert, G. R. Stark, and I. M. Kerr.** 1988. Interferon response element of the human gene 6-16. EMBO J. **7:**85–92.
60. **Qu, L., and D. Rowe.** 1992. Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J. Virol. **66:**3715–3724.
61. **Reeves, R., and A. P. Wolffe.** 1996. Substrate structure influences binding of

the non-histone protein HMG-I(Y) to free and nucleosomal DNA. Biochemistry **35:**5063–5074.
62. **Robertson, K. D., S. D. Hayward, P. D. Ling, D. Samid, and R. Ambinder.** 1995. Transcriptional activation of Epstein-Barr virus latency C promoter after 5-azacytidine treatment: evidence that demethylation at a single CpG site is crucial. Mol. Cell. Biol. **15:**6150–6159.
63. **Rooney, C. M., M. Rowe, L. E. Wallace, and A. B. Rickinson.** 1985. Epstein-Barr virus-positive Burkitt's lymphoma cells not recognized by virus-specific T-cell surveillance. Nature **317:**629–631.
64. **Rowe, D. T., M. Rowe, G. I. Evan, L. Wallace, P. J. Farrell, and A. B. Rickinson.** 1986. Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. EMBO J. **5:**2599–2607.
65. **Ruffner, H., L. F. L. Reis, D. Naf, and C. Weissmann.** 1993. Induction of type I interferon genes and interferon-inducible genes in embryonal stem cells devoid of interferon regulatory factor 1. Proc. Natl. Acad. Sci. USA **90:**11503–11507.
66. **Rutherford, M. N., G. E. Hannigan, and B. R. G. Williams.** 1988. Interferon-induced binding of nuclear factors to promoter elements of the 2-5A synthetase gene. EMBO J. **7:**751–759.
67. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
68. **Sample, J., E. B. D. Henson, and C. Sample.** 1992. The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. J. Virol. **66:**4654–4661.
69. **Schaefer, B. C., M. Woisetschlaeger, J. L. Strominger, and S. H. Speck.** 1991. Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **88:**6550–6554.
70. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** 1995. The Epstein-Barr virus *Bam*HI F promoter is an early lytic promoter: lack of correlation with EBNA 1 gene transcription in group I Burkitt lymphoma cell lines. J. Virol. **69:**5039–5047.
71. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** 1995. Redefining the Epstein-Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription initiation site in group I Burkitt lymphoma cell lines. Proc. Natl. Acad. Sci. USA **92:**10565–10569.
72. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** 1996. A simple reverse transcriptase PCR assay to distinguish EBNA1 gene transcripts associated with type I and II latency from those arising during induction of the viral lytic cycle. J. Virol. **70:**8204–8208.
73. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** 1997. Host cell-determined methylation of specific Epstein-Barr virus promoters regulates the choice between distinct viral latency programs. Mol. Cell. Biol. **17:**364–377.
73a. **Schaefer, B. C., J. L. Strominger, and S. H. Speck.** Unpublished data.
74. **Shin, W. S., Y.-H. Hong, H.-B. Peng, R. De Caterina, P. Libby, and J. Liao.** 1996. Nitric oxide attenuates vascular smooth muscle activation by interferon-γ: the role of constitutive NF-κB activity. J. Biol. Chem. **271:**11317–11324.
75. **Speck, S. H., and J. L. Strominger.** 1989. Transcription of Epstein-Barr virus in latently infected, growth transformed lymphocytes. Adv. Viral Oncol. **8:**133–150.
76. **Sung, N. S., J. Wilson, M. Davenport, N. Sista, and J. S. Pagano.** 1994. Reciprocal regulation of the Epstein-Barr virus *Bam*HI-F promoter by EBNA-1 and an E2F transcription factor. Mol. Cell. Biol. **14:**7144–7152.
77. **Takada, K., K. Horinouchi, Y. Ono, T. Aya, M. Osato, M. Takahashi, and S. Hayasaka.** 1991. An Epstein-Barr virus-producer line Akata: establishment of the cell line and analysis of viral DNA. Virus Genes **5:**147–156.
78. **Tamura, T., M. Ishihara, M. S. Lamphier, N. Tanaka, I. Oishi, S. Aizawa, T. Matsuyama, T. W. Mak, S. Taki, and T. Taniguchi.** 1995. An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. Nature **376:**596–599.
79. **Tanaka, N., T. Kawakamai, and T. Taniguchi.** 1993. Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. Mol. Cell. Biol. **13:**4531–4538.
80. **Tanaka, N., M. Ishihara, M. Kitagawa, H. Harada, T. Kimura, T. Matsuyama, M. S. Lamphier, S. Aizawa, T. W. Mak, and T. Taniguchi.** 1994. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell **77:**829–839.
81. **Taniguchi, T., H. Harada, and M. Lamphier.** 1995. Regulation of the interferon system and cell growth by the IRF transcription factors. J. Cancer Res. Clin. Oncol. **121:**516–520.
82. **Thanos, D., and T. Maniatis.** 1992. The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. Cell **71:**777–789.
83. **Thanos, D., and T. Maniatis.** 1995. Identification of the rel family members required for virus induction of the human beta interferon gene. Mol. Cell. Biol. **15:**152–164.
84. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. Cell **83:**1091–1100.
85. **Tierney, R. J., N. Steven, L. S. Young, and A. B. Rickinson.** 1994. Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. J. Virol. **68:**7374–7385.
86. **van Wijnen, A. J., F. M. I. van den Ent, J. B. Lian, J. L. Stein, and G. S. Stein.** 1992. Overlapping and CpG methylation-sensitive protein-DNA interactions at the histone H4 transcriptional cell cycle domain: distinctions between two human H4 gene promoters. Mol. Cell. Biol. **12:**3273–3287.
87. **van Wijnen, A. J., F. Aziz, X. Grana, A. De Luca, R. K. Desai, K. Jaarsveld, T. J. Last, K. Soprano, A. Giordano, J. B. Lian, J. L. Stein, and G. S. Stein.** 1994. Transcription of histone H4, H3, and H1 cell cycle genes: promoter factor HiNF-D contains CDC2, cyclin A, and a RB-related protein. Proc. Natl. Acad. Sci. USA **91:**12882–12886.
88. **Vaughan, P. S., F. Aziz, A. J. van Wijnen, S. Wu, H. Harada, T. Taniguchi, K. Soprano, J. L. Stein, and G. S. Stein.** 1995. Activation of a cell-cycle-regulated histone gene by the oncogenic transcription factor IRF-2. Nature **377:**362–365.
89. **Veals, S. A., T. Santa Maria, and D. E. Levy.** 1993. Two domains of ISGF3γ that mediate protein-DNA and protein-protein interactions during transcription factor assembly contribute to DNA-binding specificity. Mol. Cell. Biol. **13:**196–206.
90. **Weis, L., and D. Reinberg.** 1992. Transcription by RNA polymerase II: initiator-directed formation of transcription competent complexes. FASEB J. **6:**3300–3309.
91. **Weiss, A., R. L. Wiskocil, and J. D. Stobo.** 1984. The role of T3 surface molecules in the activation of human T cells: a two stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. J. Immunol. **133:**123–128.
92. **Weisz, A., P. Marx, R. Sharf, E. Appella, P. H. Driggers, K. Ozato, and B.-Z. Levi.** 1992. Human interferon consensus sequence binding protein is a negative regulator of enhancer elements common to interferon-inducible genes. J. Biol. Chem. **267:**25589–25596.
93. **Whitley, M. Z., D. Thanos, M. A. Read, T. Maniatis, and T. Collins.** 1994. A striking similarity in the organization of the E-selectin and beta interferon gene promoters. Mol. Cell. Biol. **14:**6464–6475.
94. **Woisetschlaeger, M., J. L. Strominger, and S. H. Speck.** 1989. Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **86:**6498–6502.
95. **Woisetschlaeger, M., C. N. Yandava, L. A. Furmanski, J. L. Strominger, and S. H. Speck.** 1990. Promoter switching in Epstein-Barr virus during the initial stages of infection. Proc. Natl. Acad. Sci. USA **87:**1725–1729.
96. **Wu, L., B. J. Morley, and R. D. Campbell.** 1987. Cell-specific expression of the human complement protein factor B gene: evidence for the role of distinct 5′-flanking elements. Cell **48:**331–342.
97. **Yamamoto, H., M. S. Lamphier, T. Fujita, T. Taniguchi, and H. Harada.** 1994. The oncogenic transcription factor IRF-2 possesses a transcriptional repression and a latent activation domain. Oncogene **9:**1423–1428.
98. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr virus genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
99. **Yates, J., N. Warren, and B. Sugden.** 1985. Plasmids derived from Epstein-Barr virus replicate stably in a variety of mammalian cells. Nature **313:**812–815.
100. **Zech, L., U. Haglund, K. Nilsson, and G. Klein.** 1976. Characteristic chromosomal abnormalities in biopsies and lymphoid cell lines from patients with Burkitt and non-Burkitt lymphomas. Int. J. Cancer **17:**47–56.