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Activation of the Epstein-Barr Virus DNA Polymerase Promoter by the BRLF1 Immediate-Early Protein Is Mediated through USF and E2F

CHUNNAN LIU,[1,2] NIRUPAMA D. SISTA,[1,2] AND JOSEPH S. PAGANO[1,2,3]*

Departments of Microbiology and Immunology[1] and Medicine,[3] and UNC Lineberger Comprehensive Cancer Center,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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The Epstein-Barr virus (EBV) DNA polymerase (pol) is essential for the replication of viral genomes during productive EBV infection. We have previously reported that the EBV DNA pol promoter, which is TATA-less and constitutively inactive, is activated by a genomic clone expressing both immediate-early viral transactivators, BZLF1Z and BRLF1 (R), in EBV-infected lymphoid cells. Here we demonstrate that R alone is sufficient to activate the pol promoter in EBV-negative B cells. Unlike other early promoters to which the R protein binds directly, its effect on the pol promoter does not appear to involve a direct DNA-binding mechanism. Instead, we found that two cellular transcription factors, an upstream stimulatory factor USF, and a member of the E2F family of proteins, bind directly to the pol promoter at positions −795 to −786 and −186 to −170, respectively, regions previously identified as important for activation of the pol promoter. These two sites contribute to or are essential for transactivation of the pol promoter by R in EBV-noninfected B cells. These data suggest that the R immediate-early protein may activate a key early EBV promoter (pol) through both USF and E2F.

Epstein-Barr virus (EBV) infection can exist in either a latent or cytolytic state. The latter infection state is necessary for production of virus; in addition, viral reactivation from the latent state accompanies or precedes some of the most important EBV-associated diseases, such as nasopharyngeal carcinoma and lymphoproliferative disease in immunocompromised patients.

The EBV immediate-early activators of latent infection, the BZLF1 (Z) and BRLF1 (R) gene products, have been much studied. Both Z and R are sequence-specific DNA-binding proteins which transactivate responsive genes through their respective binding sites. Transcription from the BZLF1 promoter (Zp) expresses a message encoding the BZLF1 gene product, whereas transcription from the BRLF1 promoter (Rp) gives rise to two bicistronic messages encoding both BRLF1 and BZLF1 proteins as well as a third mRNA encoding the RAZ product (29, 60). Neither Zp nor Rp has detectable constitutive levels of activity in B cells (49, 93). Both Zp and Rp are autoregulated (27, 93). There are Z-responsive elements (ZREs) in Zp that are important for its activity (27). However, although there are ZREs in Rp, R alone can activate this promoter (80, 93). Although there are parallelisms in the activations of Zp and Rp, the unique feature of Rp is that the autoregulatory effect of R is not through a direct DNA-binding mechanism.

A number of early viral promoters, such as BMLF1, BMRF1, and the DL/DR region containing both BHLF1 and BHRF1 promoters, are activated by Z or R alone or by R and Z acting synergistically (11, 14, 15, 17, 31, 40, 44, 48, 49, 68, 71). Despite the differences in activation of these early promoters, the common features are that all of these promoters have ZREs and R-responsive elements (RREs) and that both the Z and R activators bind directly to these responsive elements for activation.

Much less is known about the mechanism of activation of the EBV DNA polymerase (pol) gene, the keystone of productive viral replication. Activation mechanisms of the pol promoter might be similar to those used by immediate-early and other early promoters or might have distinctive features. We have found that the pol promoter is TATA-less with tightly clustered start sites spanning 5 bp that have been mapped in several different cell types. In fact, this is the only TATA-less early promoter that has been studied extensively, and the mechanism used to activate it may therefore differ from the other EBV promoters. The pol promoter is constitutively inactive in both lymphoid and epithelial cells, but it can be activated by a genomic expression clone for both immediate-early gene products, R and Z (28). In this report we show that unlike other early genes, which are activated by direct binding of R and/or Z, the EBV pol promoter is effectively activated by the R gene product alone through an indirect mechanism.

Since latency is likely to be dictated by cellular factors as well as by viral latency genes, we have also explored whether release of the pol promoter from its constitutively inactive state requires participation of cellular factors that activate the promoter.

Promoters or enhancers of other EBV cytolytic genes require cellular transcription factors to regulate their activities. One example is the EBV ori-Lyt enhancer which has an upstream stimulatory factor (USF)-binding site important for its constitutive activity (73). In addition, to become functional, the autoregulatory *Bam*HI F and Q promoters (Fp and Qp) for EBNA1, which are active in the cytolytic cycle (Fp) or in type 1 latency (Qp) and in epithelial cells, use an E2F family member to overcome repression of transcription produced by EBNA1 (63a, 77a, 86). The immediate-early varicella-zoster virus (VZV) gene product IE62 transactivates the VZV DNA pol promoter through a USF-binding site (62).

In this report we show that in addition to the viral protein R, the cellular proteins USF and E2F are recruited for activation of the pol promoter. Rather than binding directly to the pol promoter, as occurs with the other early promoters, BMLF1,

* Corresponding author. Phone: (919) 966-3036. Fax: (919) 966-3015.

BMRF1, and BHRF1/BHLF1, R appears to act indirectly either together with or through E2F and USF.

MATERIALS AND METHODS

Cell lines. Raji and Akata are latently EBV-infected B-lymphocyte lines. DG75 is an EBV-negative Burkitt's lymphoma B-cell line. NPC-KT is an EBV-positive epithelial cell line, and ADAH is an EBV-negative epithelial cell line. All lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Epithelial cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum.

Plasmid constructs. The construction of plasmid pPOLCAT has been described previously (28). The internal deletion mutants were constructed by restriction digestion and religation. Site-directed mutants of pPOLCAT were made with the Bio-Rad Muta-Gene phagemid in vitro mutagenesis kit on the basis of the Kunkle method (53). The expression constructs of EBV immediate-early proteins have been described elsewhere (68). The pCMV-Z plasmid contains the BZLF1 cDNA fragment under the control of the cytomegalovirus immediate-early promoter in the pGem2-based vector (pHD1013). The pCMV-R plasmid is derived from a *Hin*dIII-*Hin*dIII fragment from a genomic expression construct, pCMV-RZ, such that the BRLF1 gene is linked to the cytomegalovirus immediate-early promoter in a pUC-18 vector.

Transfections and chloramphenicol acetyltransferase assays. Plasmid DNA was purified through Qiagen columns. For each transfection, 10^7 cells were electroporated at 1,500 V with the University of Wisconsin Zapper electroporation unit. Cells were then suspended in 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 48 h at 37°C in 5% CO_2. Cell extracts were prepared by washing cell pellets twice in phosphate-buffered saline (PBS) solution and resuspending them in 200 μl of 0.25 M Tris-HCl (pH 7.5), followed by four cycles of freezing and thawing. Chloramphenicol acetyltransferase activity was assayed as described previously by incubating each sample with acetyl coenzyme A and [^{14}C]chloramphenicol at 37°C for 2 h. Acetylated reaction products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by scanning on a Molecular Dynamics PhosphorImager. Percent acetylation and fold activation were calculated as previously described (28).

Preparation of GST fusion proteins. The construction of the bacterial expression construct glutathione *S*-transferase (GST)-R has previously been described (68). Bacteria containing GST-R plasmid were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30°C. Cells were then pelleted and lysed in 50 mM KCl–8 μM leupeptin–0.5 mM phenylmethylsulfonyl fluoride by sonication. Lysates were cleared by centrifugation to remove cell debris.

Preparation of cell extracts. Whole-cell extracts were prepared from Raji, Akata, Daudi, Louckes, NPC-KT, and ADAH cells. In each case, 5×10^6 cells were washed with cold PBS and resuspended in 0.5 ml of whole-cell lysis buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0), 250 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, 0.1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. Cells were frozen and thawed three times, and cell debris was removed by centrifugation. Protein concentrations of the cell lysates were determined by the Bradford method and stored in aliquots at −70°C.

Electrophoretic mobility shift assays. DNA probes were generated by either restriction enzyme digestion or annealing oligonucleotides spanning the regions of interest and by filling in the single-strand overhangs with [^{32}P]dATP and/or [^{32}P] dCTP using the Klenow enzyme.

The general binding reaction mixture with whole-cell extracts contained 10 mM Tris-HCl (pH 8.0), 6 mM KCl, 0.1% Nonidet P-40, 10% glycerol, 2% Ficoll, 0.05 mg of bovine serum albumin per ml, and 0.05 mg of sonicated salmon sperm DNA per ml. The cell extracts were incubated with probes (2×10^4 cpm) for 20 min at room temperature. The bound complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.25× Tris-borate-EDTA buffer.

The modified USF-binding reactions were performed in 10 mM Tris (pH 7.4)–50 mM NaCl–1 mM EDTA–5% glycerol–4 mM dithiothreitol–0.1 mg of poly(dI-dC) · poly(dI-dC) per ml.

The R-binding reactions (68) were performed in 10 mM HEPES (pH 7.9)–5 mM $MgCl_2$–130 mM KCl–5% Ficoll–0.5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–0.1 mg of poly(dI-dC) · poly(dI-dC) per ml at 4°C.

For the supershift assays, whole-cell extracts were incubated with antibodies for 15 min at room temperature before incubation with probes for another 20 min.

Competitors used in mobility shift assays. Oligonucleotides AP1, SP1, OCT1, GRE, CREB, and NF-κB are from Promega. The rest of the oligonucleotide competitors were synthesized in the Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility. The sequences for the different E2F sites are as follows: human dihydrofolate reductase (DHFR) promoter, 5′-CCACAATT TCGCGCCAAACTTGAC-3′; adenovirus type 5 E2 promoter (E2), 5′-CGT AGTTTTCGCGCTTAAATTTGAGAAAGGGGCGCGAAACTAGTC-3′; human c-*myc* promoter (c-*myc*), 5′-CAGAGGCTTGGCGGGAAAAAGAACG GAGGGAGGGATCGCGCTGAGTA-3′; human thymidine kinase promoter (tk), 5′-TCGACGGGGCGGGCTGCGGCAAATCTCCCGCCAGTCAGCGG CCGGGCGCTGATTGGCCC-3′; and the EBV *Bam*HI F promoter (Qe), 5′-GTGCGCTACCGGATGGCGGGTAATACA-3′.

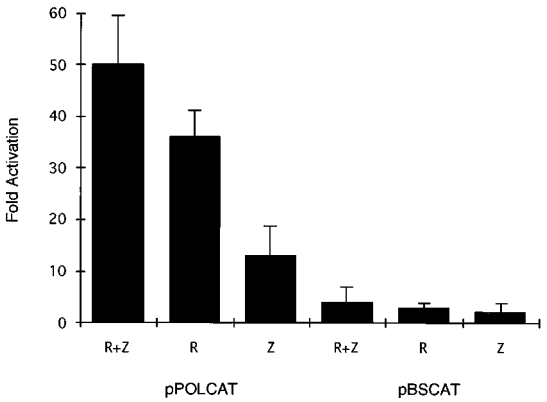

FIG. 1. Effect of the EBV immediate-early R and Z transactivators on the EBV DNA pol promoter. pPOLCAT plasmid (5 μg) or the promoterless negative control plasmid pBSCAT were cotransfected with 5 μg of either pCMV-R (R), pCMV-Z (Z), pCMV-R plus pCMV-Z (R+Z), or pHD1013, a negative control vector, into the EBV-negative cell line DG75. The total amounts of transfected plasmids were increased to 10 μg with pHD1013. Results are expressed as fold activation over baseline activity with pHD1013.

Methylation interference. Mapping of protein binding to probes by methylation interference was performed as previously described (79). DNA probe (10^5 cpm) was incubated with 30 μg of Raji whole-cell extract in 5 volumes of the binding reaction mixture used for mobility shift assays. After electrophoretic mobility shift assay, the bound and free DNA probes were excised and electroeluted from the gel pieces. The eluted probes were incubated with 100 μl of 1 M piperidine at 90°C for 30 min. The probes were then washed and precipitated. Equal amounts of the radioactive bound and free probes were analyzed on 8% polyacrylamide sequencing gels and visualized by autoradiography.

RESULTS

The EBV DNA pol promoter is primarily R responsive. We have reported that EBV DNA pol promoter activity resides in a 1.29-kb genomic fragment that can be activated in EBV-infected lymphoid cells by cotransfection with a genomic expression construct, pCMV-RZ, which expresses both EBV immediate-early gene products R and Z (28). Here, we examine the effects of R and Z separately in EBV-negative cells. The pPOLCAT construct was transfected into DG75 lymphoid cells along with either the vector (pHD1013) or expression clones for Z and R alone or together. As shown in Fig. 1, the pol promoter was activated efficiently by the R-expression clone. Z had a modest effect in activating the promoter compared with R (Fig. 1). This result is reminiscent of the slight effect of Z on the BMLF1 and BMRF1 early promoters in certain EBV-negative lymphoid cells (44). With the pol promoter, when both R and Z transactivators were coexpressed, the stimulatory effect on the promoter activity was additive. Therefore, the predominant viral activator for the pol promoter appears to be R.

Binding of R to the pol promoter was not detected. R is a sequence-specific DNA-binding protein that binds to the RREs in the EBV BMRF1, BHRF1, BHLF1, and BMLF1 early promoters (31, 34, 36, 68). To determine whether R activation of the pol promoter involves direct binding of the R protein, mobility shift assays using bacterially expressed GST-R fusion protein (a gift from Shannon Kenney) were performed. We used a series of restriction-digestion fragments that span the pol promoter sequence (Fig. 2A) as probes and a positive control probe from the BMLF1 promoter. The R protein bound to the BMLF1 control probe (Fig. 2B, lane 3) specifically, as shown by competition with an oligonucleotide containing an R-binding site from the BMLF1 promoter (lane

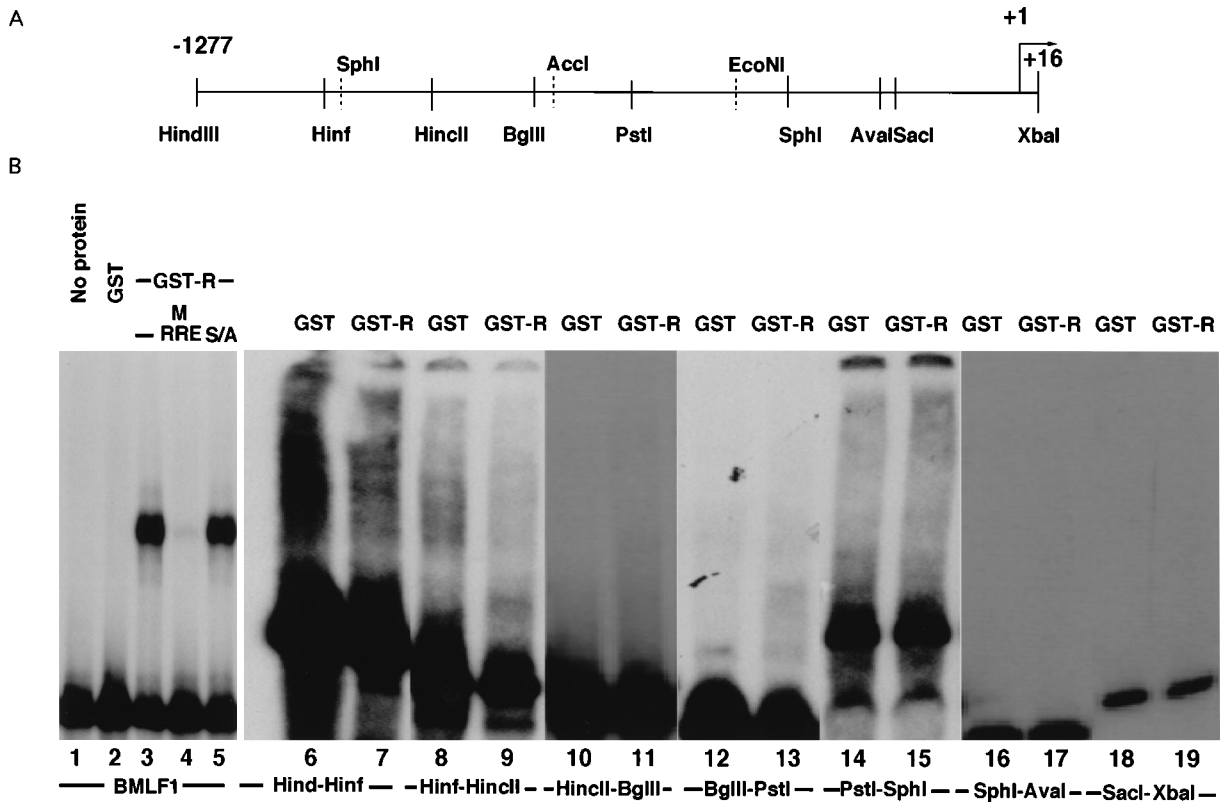

FIG. 2. Binding of the R protein directly to the pol promoter is not detected. (A) Restriction enzyme digestion fragments from the pol promoter. The solid vertical lines represent the cut sites for the enzymes, the names of which are indicated. The vertical dashed lines define additional fragments tested (data not shown). (B) Probes were ^{32}P end labelled and incubated with either bacterial R protein (GST-R) or GST protein (lanes 6 through 19). A 270-bp probe from the BMLF1 promoter containing a known R-binding site was used as the positive control (lanes 1 through 5). Binding reaction mixtures were resolved on a 4% nondenaturing polyacrylamide gel. The results are shown. Competition of the bound complex in lane 3 with a 200-fold excess of unlabelled oligomer (M) containing the R-binding site from the BMLF1 promoter is shown in lane 4, while competition with a nonspecific oligomer (S/A) is shown in lane 5. The added proteins are indicated above each lane.

4). However, binding of the R protein to any pol promoter sequences was not detected under the conditions tested (Fig. 2B, lanes 6 through 19), nor were consensus R-binding sequences found. We have also used a different set of restriction enzymes as indicated by the dashed lines in Fig. 2 to generate probes which overlap the restriction sites used previously. We did not detect any R binding to the pol promoter with these probes (data not shown). Therefore, it appears that as reported for other promoters (37, 69, 93), activation of the pol promoter by R is not mediated through direct binding of the protein.

Detection of protein-DNA complexes binding to the H/B and S/A elements. Two regions of the pol promoter that are important for its activity have been identified by 5′ deletional analyses (28). One is a 140-bp region between *Hin*cII and *Bgl*II (H/B), and the other is the 101-bp region between *Sph*I and *Ava*I (S/A). Since R did not appear to bind to the pol promoter directly, we investigated whether activation by R may be mediated through cellular factors. To determine whether cellular proteins contribute to activation of the promoter, we first used mobility shift assays to search for specific DNA-binding complexes formed from crude whole-cell extracts of uninduced or induced Akata cells. The results are shown in Fig. 3. Specific DNA-binding complexes were detected with both the H/B and S/A probes with extracts from uninduced (Fig. 3A and B, lanes 1) and induced (Fig. 3C and D, lanes 1) cells. The specificities of the DNA-protein complexes were tested by the addition of 100-fold excesses of unlabeled probe (Fig. 3A to D, lanes 2) or an array of nonspecific competitors, as indicated above the lanes in Fig. 3. Only the unlabelled probes could specifically interfere with complex formation. The S/A probe did not interfere with binding to the H/B probe, nor did the H/B probe interfere with binding to the S/A probe (data not shown). Furthermore, these complexes were detected regardless of induction of viral cytolytic cycle replication by surface immunoglobulin cross-linking.

Both H/B and S/A bind to ubiquitous cellular protein. To determine whether these specific complexes were of viral or cellular origin and whether they are B-cell specific or exist in both B cells and epithelial cells, whole-cell extracts from a variety of cell types were used in mobility shift assays. DG75 is an EBV-negative B-cell line. Akata and Raji are both latently EBV-infected B-cell lines. ADAH is an EBV-negative epithelial cell line, whereas NPC-KT is a fusion between ADAH and an EBV-positive nasopharyngeal carcinoma. As shown in Fig. 4, all cell extracts exhibited similar complex formation profiles, indicating that ubiquitous cellular proteins bind to both the H/B and S/A elements. The binding specificities of these complexes were tested by inhibition with 100-fold excesses of unlabelled probe (Fig. 4, lanes 7 and 14).

Mapping of the binding sites of the cellular protein. To identify the binding sequences of these protein complexes, methylation-interference assays were performed with the protein complexes from Raji whole-cell extracts. As shown in Fig. 5, protected regions were detected in both the H/B (Fig. 5A) and S/A (Fig. 5B) probes. Examination of the protected 10-bp sequence (−795 to −786) in H/B revealed an E-box CA--TG

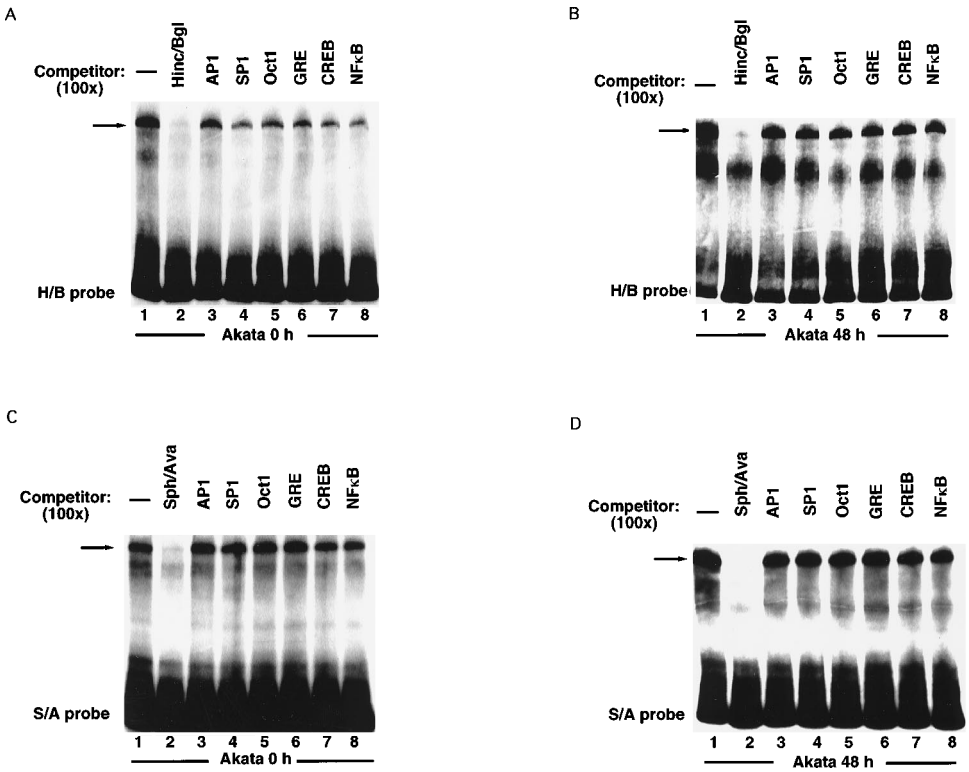

FIG. 3. Detection of protein complexes bound to the pol promoter fragments H/B and S/A. Mobility shift assays were performed to detect complexes from uninduced (0 h) or anti-immunoglobulin G-induced (48 h) Akata whole-cell extracts with ^{32}P-end-labelled probes H/B (A and B) and S/A (C and D). The unlabelled probe competitor and a panel of oligonucleotide competitors with known DNA-binding motifs are indicated above each lane. The arrows indicate specific complexes.

motif to which basic helix-loop-helix proteins, such as the cellular transcription factors USF, MYC, and TFE3/TFEB, are capable of binding (5, 7, 81). In probe S/A, a 17-bp protected region (−186 to −170) contained the sequence 5′-GGCG CACGCGCC-3′. A sequence homology search did not disclose obvious homology to known transcription factor-binding sites.

Oligomeric probes from the mapped binding sites exhibit the same protein-binding activity. To confirm that the meth-

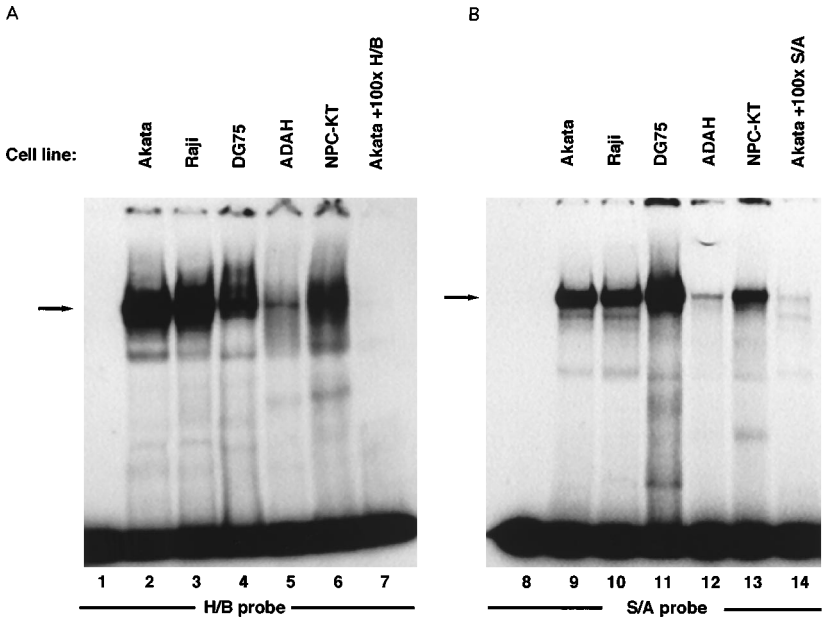

FIG. 4. Ubiquitous cellular complexes bind to H/B and S/A probes. H/B (A) and S/A (B) fragments were ^{32}P end labelled, and gel mobility shift assays were performed with whole-cell extracts from a variety of cell lines. The sources of the whole-cell extracts are indicated above the lanes. DG75 is an EBV-negative lymphoid cell line. Akata and Raji are latently infected EBV lymphoid cells. ADAH is an EBV-negative epithelial cell line. NPC-KT is an EBV-infected epithelial cell line. Arrows indicate specific complexes. Specificities of protein binding were tested by competition with unlabelled probes with Akata extracts (lanes 7 and 14).

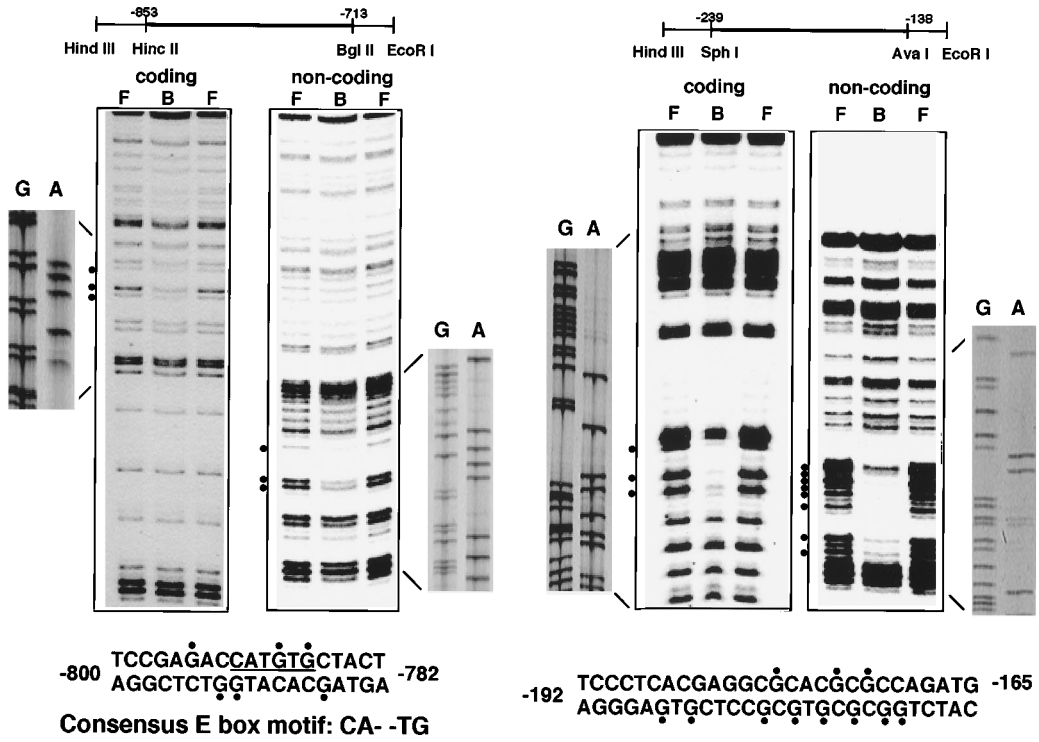

FIG. 5. Mapping of the cellular protein-binding sites in the H/B and S/A regions by methylation interference. H/B and S/A fragments were ^{32}P end labelled on either the coding or noncoding strands. Labelled probes were partially methylated with dimethylsulfate and incubated with Raji whole-cell extracts. Complexes were resolved by gel electrophoresis. Bands representing bound (B) and free (F) probes were removed separately, cleaved with piperidine, and analyzed on an 8% polyacrylamide–urea sequencing gel. Missing guanine residues representing positions of protein interaction are marked with filled circles. To the side of each panel is the G+A ladder around the protected region. The sequence in the regions of protection is shown below each gel. The sequence in the H/B probe containing an E-box motif is underlined.

ylation-interference assay had identified the protein-binding sites in the H/B and S/A probes, oligonucleotide probes that corresponded to the mapped sequences were synthesized. Mobility shift assays were done as described above for the full-length probes. Whole-cell extracts were from uninduced and induced Akata cells. Both oligomeric probes formed specific DNA-protein complexes (Fig. 6B, gels a and b, lanes 1 and 4). Specificity was demonstrated both by competition with 100-fold excesses of unlabelled probe (Fig. 6B, gels a and b, lanes 2 and 5) and by lack of competition with the mutated oligomer (Fig. 6B, gels a and b, lanes 3 and 6) and other unrelated oligomers used in the experiment shown in Fig. 3 (data not shown). Moreover, these two oligomers were able to compete with the binding activities formed with the full-length probes (Fig. 6C). Therefore, cellular protein-binding activity was localized to a 10-bp region between −795 and −786 upstream of the transcriptional start site and to a 17-bp sequence between positions −186 and −170.

USF binds to the H/B probe. The E-box motif between −795 and −786 is recognized by USF in the transactivation of both viral and cellular promoters (22, 62, 66, 70, 73, 76). We tested whether the USF protein bound to positions −795 and −786. With a 32-bp oligonucleotide that included the protected area as the probe, two closely migrating bands were detected, indicating the presence of both forms of USF proteins (81) in Raji cell extracts (Fig. 7B, lane 2). A cold oligonucleotide containing the USF-binding site from the adenovirus major late promoter (Fig. 7B, lane 5) specifically inhibited the formation of both bands, but a nonspecific SP1 oligonucleotide (Promega) did not (Fig. 7B, lane 6). A specific rabbit polyclonal antibody (a gift of Michelle Sawadogo) which recognizes both USF1 and USF2 (antiUSF43) disrupted the formation of the bands from their normal positions. Instead, other more slowly migrating bands were detected (Fig. 7B, lane 3). Normal rabbit serum had no effect on complex formation (Fig. 7B, lane 4). Moreover, an oligonucleotide with a mutated USF-binding site (GATGTG) did not compete with the formation of these complexes (Fig. 6B, gel a, lanes 3 and 6). As a positive control, we performed a mobility shift assay with an authentic USF oligonucleotide probe; the result, shown in Fig. 7C, was identical to that obtained with the H/B oligonucleotide pol promoter probe (Fig. 7B). Therefore, it appears that a USF family member protein binds to the E box between −795 and −786.

An E2F-like factor binds to the pol promoter. Since E2F binds to and activates another EBV promoter (86), Fp, which may function as a cytolytic cycle promoter (55, 74, 77, 78, 83), we tested whether E2F could bind to the pol promoter with the 101-bp restriction fragment S/A as a probe. The binding activity detected earlier in Raji cell extracts was efficiently inhibited by a 27-bp oligonucleotide competitor, Qe, originating from Fp and shown previously to bind to an E2F-like protein (86) (Fig. 8A, lane 5). Oligonucleotides containing two other known E2F-binding sites from the human thymidine kinase promoter and from the adenovirus E2 promoter could partially inhibit the complex formation (Fig. 8A, lanes 7 and 9). As with Fp, an oligonucleotide containing the E2F site from the DHFR promoter did not inhibit complex formation (Fig. 8A, lane 10). The E2F-binding site from the human c-*myc* promoter did not inhibit the complex formation in the pol promoter (Fig. 8A, lane 8) but could inhibit the complex formed with Fp. Two oligonucleotides containing either the mapped sequences from

the H/B region or an SP1 site were used as nonspecific competitors (Fig. 8A, lanes 4 and 6).

To verify that an E2F-like protein existed in the complex, E2F-specific polyclonal antibody (a gift of Scott Hiebert) was used. As shown in Fig. 8B, protein complex formation on S/A was blocked by the anti-E2F antibody (lane 3) but not by a polyclonal antibody against EBV BMLF1 protein (a gift of Hans Wolf) (lane 4). This anti-E2F antibody also blocked complex formation on the adenovirus E2 probe (Fig. 8B, lane 12). This blocking specificity was further demonstrated by the inability of the anti-E2F antibody to block the protein complex formed on a control probe from the EBV *Bam*HI C EBNA promoter (Fig. 8B, lane 7).

These data indicate that an E2F-like protein binds to the proximal *cis*-acting element S/A. The mapped binding site contains GGCGC and CGCGC sequences, both of which are in several functional E2F-binding sites, such as the one in the DNA pol α promoter (88).

Mutation of either the USF-binding site or the E2F-binding site decreases transactivation of the pol promoter by R. To address whether USF or E2F binding to the promoter has any functional impact on promoter responsiveness to the R transactivator, three constructs, pPOLCATΔUSF, pPOLCATΔE2F, and pPOLCATΔΔ, were made by site-directed mutagenesis of pPOLCAT, in which either the USF- or E2F-binding site or both were deleted. The E box was specifically replaced by the mutation GATGTG (pPOLCATΔUSF), and in the pPOLCAT ΔE2F construct the E2F-binding site GGCGCACGCGCC was mutated to GGCGCTCTAGAC. The corresponding mutated oligomers did not inhibit the binding of USF or E2F, as shown in Fig. 6B (gels a and b, lanes 3 and 6), and probes containing these mutations did not bind to USF or E2F (data not shown). The levels of activation by R in DG75 cells were decreased about 50% when either the USF-binding site or the E2F-binding site was mutated, as shown in Fig. 9. The double mutation produced a 20% further decrease of the promoter activity level (down to 30% that of the wild type). This result indicates that both the E box in H/B and the E2F site in S/A indeed are important but not sufficient to activate the R responsiveness of the pol promoter.

DISCUSSION

EBV replicates by two entirely different mechanisms during latent and virus-productive infection. In productive EBV infection, the EBV DNA pol is used to replicate large numbers of linear viral genomes. In latent infection, the EBV pol gene is constitutively inactive and EBV episomes are replicated by host-cell enzymes. Tetradecanoyl phorbol acetate and other agents (19, 24, 47, 59, 64) can readily induce viral reactivation, which ultimately activates the pol gene. However, most of the studies of viral reactivation have focused on events early in the reactivation cascade, namely, activation and regulation of the immediate-early genes BZLF1 and BRLF1. Since the whole series of events is directed at activating viral replication, it is reasonable to examine the transcriptional regulation of one of the essential early replicative genes, the DNA pol gene. We had made an initial characterization of the pol promoter (28) leading to this study, which was designed to identify viral and cellular factors involved in regulating this promoter.

In this report, we have demonstrated that the viral immediate-early protein R can efficiently activate the pol promoter through a non-DNA-binding mechanism. We also show that two cellular transcription factors, USF and E2F, that are important activators of other promoters, Fp and the ori-Lyt enhancer, bind to pol promoter sequences and appear to be major contributors to its activation. Most importantly, the

A

H/B wt	CTCCGAGACCATGTGCTACTC
H/B mut	GAGGGTCCCTGGCTCCGTCTAGATGTGCTAC
S/A wt	CCCTCACGAGGCGCACGCGCC
S/A mut	TTCCCTCACGAGGCGTCTAGACAGATGTC

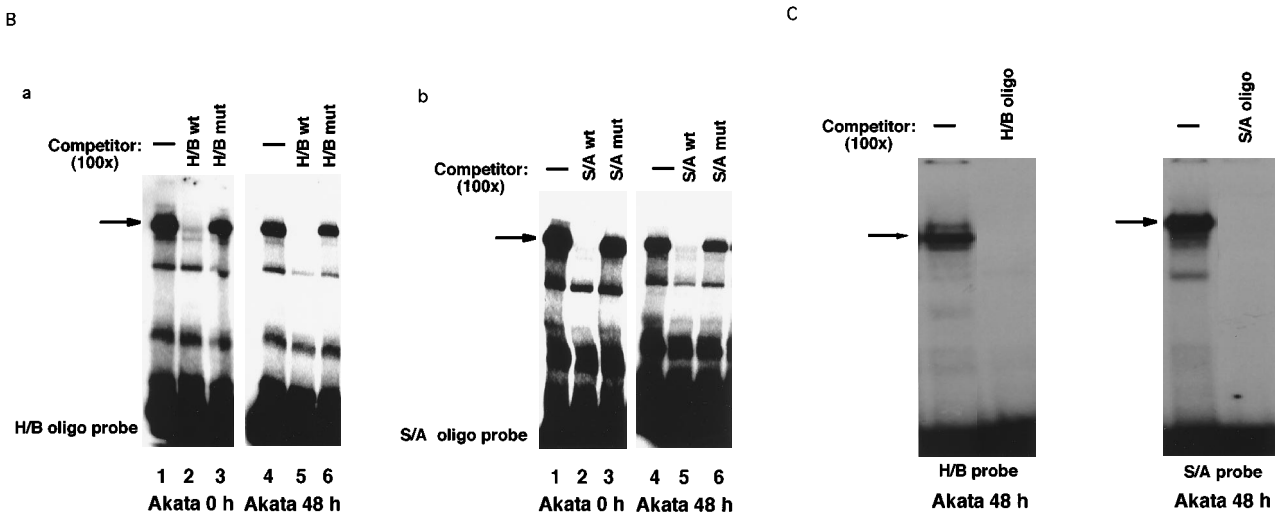

FIG. 6. Protein complexes bind to the oligonucleotide probes containing the mapped sequences from H/B and S/A. (A) DNA sequences of wild-type (wt) and mutated (mut) oligonucleotides from H/B and S/A used in gel shift assays. The mutated sequences are underlined. (B) Labelled oligonucleotide (oligo) probes H/B (gel a) and S/A (gel b) were incubated with either uninduced (0 h) or anti-immunoglobulin G-induced (48 h) Akata whole-cell extracts. (C) Full-length H/B and S/A probes were labelled and incubated with Akata whole-cell extract (48 h). Arrows indicate specific complexes. The unlabelled oligonucleotide competitors are indicated above each lane.

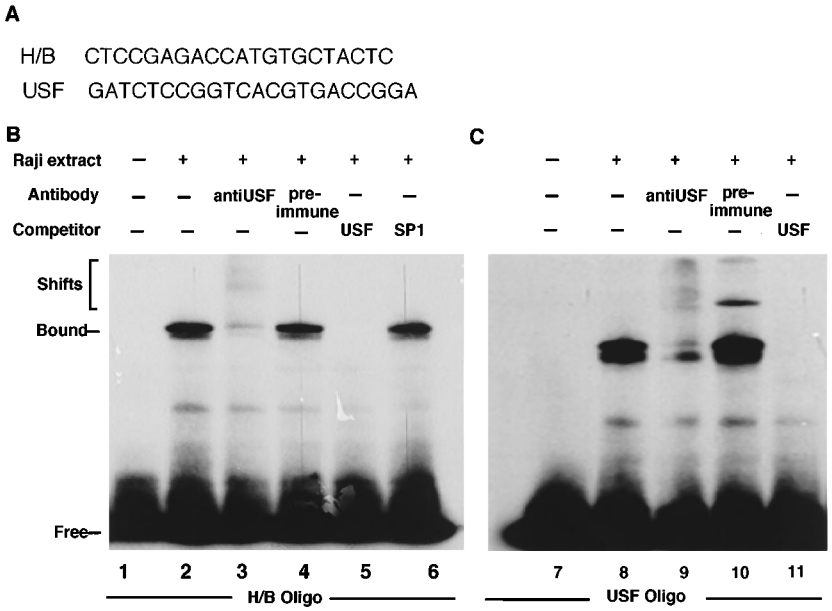

FIG. 7. USF binds to H/B. (A) DNA sequences of the probe and the authentic USF competitor used in mobility shift assays. Labelled H/B oligonucleotide (oligo) (B) or labelled USF oligonucleotide (C) were incubated with either no extract (lane 1) or Raji whole-cell extract in the absence (lanes 2 to 4) or presence (lanes 5 and 6) of unlabelled oligonucleotide competitors, 1 μl of preimmune (lane 4), or 1 μl of anti-USF43 serum (lane 3). USF-specific shift is indicated by the bracket.

binding sites of these two transcription factors were precisely mapped and overlap the R-responsive regions.

R is a 605-amino-acid protein which is expressed in the immediate-early stage of viral reactivation. It contains a DNA-binding region located in the 280 N-terminal amino acids, a dimerization domain within the 232 N-terminal amino acids, and a transcriptional activation domain localized in the C-terminal part of the protein. In vitro, R has been shown to bind to specific DNA sequences as a homodimer; dimerization is essential for DNA binding but can also occur without DNA binding (61). The transactivation domain contains two regions with structures similar to those of other transcription factors: one is a proline-rich region (amino acids 352 to 515) and the other is an acidic region (amino acids 515 to 605). The DNA-binding site repertoire for R has been characterized recently as 5′-GNCCN$_9$GGNG-3′ (36). Activation by R alone or by R and Z of responsive promoters, such as the BMLF1, BMRF1, and BHRF1/BHLF1 early promoters, is mediated through direct binding of the protein to RREs in those promoters (31, 34, 36, 68). In addition, c-*myb*, a cellular transcriptional activator, has been shown to cooperate with Z to activate synergistically the BMRF1 promoter, indicating that c-*myb* may act as a cellular functional homolog of R (50). Although R is essential to activate the pol promoter, we were unable to demonstrate direct R binding to the promoter. Moreover, computer analysis searches did not disclose RREs in the pol promoter. Thus R may activate the pol promoter by an indirect mechanism, as is apparently the case with the EBV BRLF1, the human immu-

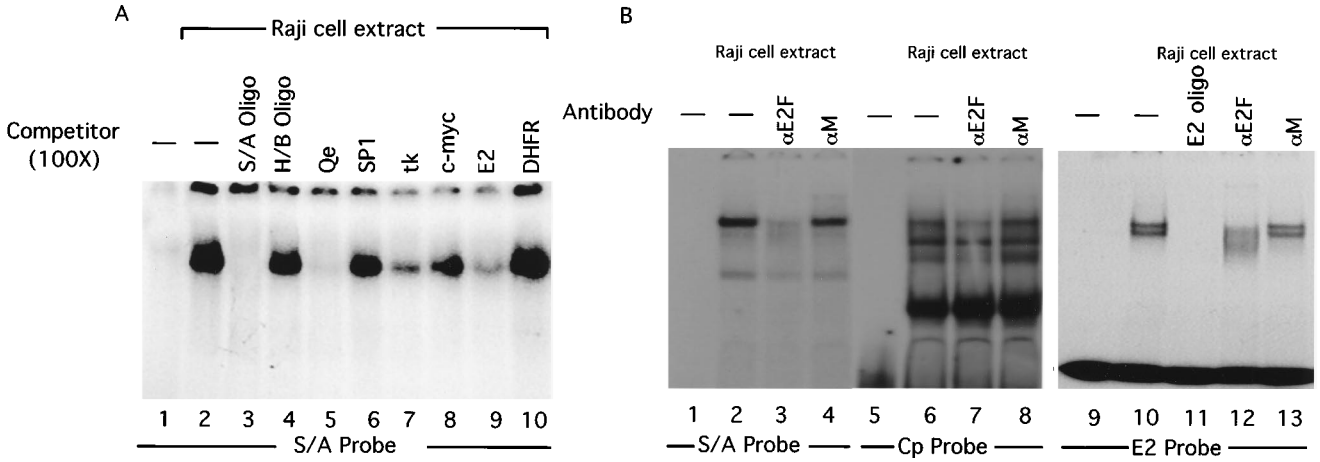

FIG. 8. An E2F-like complex binds to S/A. (A) S/A probe was incubated with either no extract (lane 1) or Raji whole-cell extract (lanes 2 to 10) and inhibited with either unlabelled S/A (lane 3), H/B (lane 4), or SP1 (lane 6) oligomer, or oligomers with E2F sites from various promoters (lane 5 and lanes 7 to 10). Oligo, oligonucleotide; tk, thymidine kinase. (B) Labelled S/A probe, a negative control probe from the *Bam*HI C promoter (Cp) of EBV, or a positive control probe from the adenovirus E2 promoter were incubated with Raji whole-cell extracts and 0.5 μl of a polyclonal antibody to either E2F (αE2F)(lanes 3, 7, and 12) or BMLF1 protein (αM) (lanes 4, 8, and 13). Specific blocking of complex formation was detected only with the S/A probe and anti-E2F antiserum (lane 3).

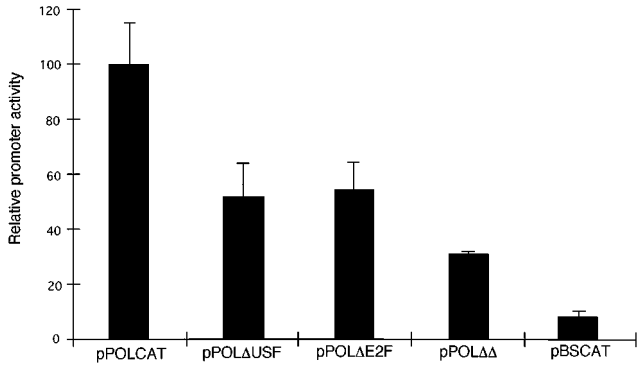

FIG. 9. The USF-binding site in H/B and the E2F-binding site in S/A are required for R transactivation. Site-directed mutants (pPOLCATΔUSF [pPOLΔUSF], pPOLCATΔE2F [pPOLΔE2F], and pPOLCATΔΔ [pPOLΔΔ]) of the pPOLCAT parent construct, in which the USF-binding site was mutated to GATGTG, the E2F-binding site was mutated from GGCGCACGCGCC to GGCGCTCTAGAC, or both sites were mutated, plus pPOLCAT and pBSCAT were cotransfected into DG75 cells with pHD1013 or pEBV-R. The results are presented as relative promoter activity of the mutated construct compared with that of the wild type after R activation.

nodeficiency virus long terminal repeat, and the human c-*myc* promoters (37, 68, 93). The intermediary proteins involved in the activation of these three promoters have not been identified, and it will be interesting to see whether activation of the BRLF1 promoter or the c-*myc* promoter is mediated by USF or E2F.

Two USFs, the 43 kDa-USF1 and the 44-kDa USF2, were first identified in HeLa cell extracts through their involvement in stimulating transcription in vitro from the adenovirus major late promoter (76). Both are ubiquitous proteins that form homo- and heterodimers both in vitro and in vivo (81, 82). USF is a member of the basic helix-loop-helix leucine zipper family of proteins, which includes Myc, Max/Myn, Mad/Mxi, AP4, and TFE3/TFEB (3, 7, 10, 65, 67). The USF1 and USF2 proteins show a high degree of homology in their C-terminal regions, the basic helix-loop-helix leucine zipper region. A core sequence, CANNTG, called the E-box motif, is recognized by all known basic helix-loop-helix leucine zipper proteins. The two forms of USF bind to the E-box motif with identical levels of affinity (81). The USF bound to its consensus recognition site upstream of the TATA box can stimulate transcription, but it can also bind to initiator elements encompassing the transcription start site and initiate transcription. Cooperative interaction between USF and the TATA-binding factor TFIID and an initiator-binding factor TFII-I has been demonstrated on the adenovirus major late promoter, which suggests that its transactivation mechanism may involve facilitation of general transcriptional machinery (72, 75). Similarly, Z interacts with TFIID in an artificial promoter which contains five copies of ZRE to facilitate the binding of the basal transcription machinery (57). Since Z has some effect on the pol promoter and Z protein binds to a ZRE proximal to the start site (data not shown), we tested whether mutation of this ZRE (located at position −40 bp) would abolish pol promoter activity, but there was little effect regardless of whether the promoter was activated by R or Z alone or together. Therefore, perhaps USF supplies this function instead of Z. However, we have not directly studied the interaction of Z with the core pol promoter transcription factors.

Putative USF-binding sites are found in many viral as well as cellular promoters. USF can activate the human growth-hormone promoter, the p53 tumor-suppressor gene promoter, as well as the human immunodeficiency virus long terminal repeat (22, 66, 70). Moreover, USF binds to an E-box motif in the EBV ori-Lyt enhancer; binding of USF to this site is a major factor contributing to constitutive enhancer activity (73). Recently, USF has been shown to cooperate with the immediate-early protein (IE62) of another herpesvirus, VZV, to activate its pol promoter. VZV IE62 is a homolog of immediate-early proteins ICP4 and IE180 of the closely related herpes simplex virus and pseudorabies virus, respectively (13, 25, 91). However, there is no information on the R homolog in these viruses. IE62 is a sequence-nonspecific DNA-binding protein with an acidic transactivation domain. In the report, transactivation of the VZV pol promoter by IE62 is mediated through the USF-binding site in the promoter, but the exact mechanism of this effect was not determined (62).

Our results show that both forms of USF bind directly to the pol promoter. Moreover, the core binding site of USF is required for R to transactivate the pol promoter. On the basis of the finding that R did not bind to the pol promoter directly, we propose that R activates the pol promoter by means of USF. R may upregulate the USF promoter to increase the supply of USF in cells, or R may activate USF by direct interaction with it. Although the mobility shift assays did not reveal differences in USF-binding activity from uninduced and induced cell extracts, it is possible that the level of induction efficiency attained was not great enough to detect an increase in functional USF in this assay. In addition to the interaction between USF and components of the general transcriptional machinery, USF has recently been demonstrated to interact with E1A, which stimulates transcription of adenovirus genes as well as a wide variety of other viral and cellular genes (58). The results show that E1A can interact with DNA-binding domains from several classes of cellular transcription factors, including ATF-2, c-jun, SP1, and USF, and can thereby be recruited to diverse promoters. R may function in a similar scenario by direct interaction with USF to transactivate the pol promoter itself or simply to serve as a bridge between USF and the basal transcriptional machinery.

Perhaps the most interesting finding is that a family member of the cellular transcription factor E2F may also mediate the R responsiveness of the pol promoter. E2F, previously identified as a component of early adenovirus transcription (51), has now been shown to be important in controlling cell proliferation. E2F is a central driver of the cell cycle and is an activator of many genes essential in S phase, including DHFR, thymidine kinase, and DNA pol α, all of which are needed for DNA synthesis (8, 18, 63). Members of the E2F family appear to be functional targets for the action of the tumor suppressor protein Rb and its related proteins (6, 9, 16, 56, 63). Five E2F-like proteins have been cloned (6, 30, 32, 41, 45, 46, 56, 84). Recent studies have shown that E2F activity is actually a combined result of the E2F family and the DP-1 family of proteins (92). The E2F-1 and DP-1 heterodimer has increased levels of DNA-binding activity and transactivation efficiency for E2F-responsive promoters (4, 42, 52). Furthermore, the association of E2F-1 and DP-1 is required for stable interaction with pRB in vivo, and transactivation by E2F-1 and DP-1 is inhibited by pRB (42). It has been shown that many DNA viral proteins, including adenovirus E1A, simian virus 40 T antigen, and the human papillomavirus E7 interact with RB or its related proteins and release functional E2F (12, 20, 23, 90). The human cytomegalovirus immediate-early transactivator protein IE2 interacts with RB and, by displacement, relieves RB-induced repression of E2F (38). For EBV, there is a report of viral nuclear protein EBNA-5 binding to RB and p53 proteins in vitro (87), but there is no in vivo evidence of the significance of

those interactions in terms of modulating E2F. We have recently shown that an E2F family member is involved in the expression of the EBV Fp (86) (and presumably Qp[63a, 77a]; Fp was the first EBV promoter identified as activated by E2F. Although Fp had been thought to be expressed in the latent phase of infection, Fp appears to be active in the cytolytic phase (55, 77). It will be interesting to test whether R activates Fp and provides the switch via E2F to the functioning of Fp in the cytolytic cycle.

The results presented here provide the first indication that the EBV immediate-early protein R may be involved in modulating E2F activity. It has been known for years that the induction of EBV lytic viral replication follows entry of the cell into S phase (39). Although many of the genes required for viral DNA replication are virally encoded, cells in S phase may have higher concentrations of intracellular nucleotide pools and other substrates which are necessary for efficient viral DNA synthesis. By inducing E2F activity, either quantitatively or qualitatively, the virus may stimulate quiescent cells into S phase. It has been shown that DHFR and thymidine kinase are activated in both adenovirus- and simian virus 40-infected cells (63). Human cytomegalovirus has also been documented to induce cellular DNA synthesis (1, 21, 85). However, it is too early to speculate on which mechanism is involved in transactivation of the pol promoter via R and E2F.

The upstream sequences of the BALF2 gene, which encodes the single-stranded DNA-binding protein, and of the BBLF4 gene, which encodes DNA primase, contain both putative E2F- and USF-binding sites. The BALF2 and BBLF4 gene products are essential EBV replication cofactors with HSV homologs (2, 26, 33, 43, 54, 89). Understanding the specific mechanism whereby the R protein activates the pol promoter may uncover potential general mechanisms for the coordination of 5′ activation of other viral replicative promoters.

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