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Reciprocal Antagonism of Steroid Hormones and BZLF1 in Switch between Epstein-Barr Virus Latent and Productive Cycle Gene Expression

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BZLF1 repression of transcription from the Epstein-Barr virus BC-R2 promoter (Cp) was shown to require a glucocorticoid response element in *cis* and glucocorticoids in *trans*. The mechanism of the repression is indirect and involves up regulation of the cellular c-*fos* proto-oncogene. Glucocorticoids maintain Epstein-Barr virus latency, and removal of glucocorticoids from the cell culture medium results in activation of the productive cycle. This inverse regulation of the expression of latent and productive cycle genes contributes to the switch between virus latency and the productive cycle. Glucocorticoid control of BC-R2 might also provide a mechanism for EBNA promoter switching during early infection and in development of the restricted latent pattern of gene expression.

Epstein-Barr virus (EBV) is a human herpesvirus that infects B lymphocytes and certain epithelial cells. It is thought to contribute to the development of many cases of Burkitt's lymphoma (BL), nasopharyngeal carcinoma, and some cases of Hodgkin's lymphoma. EBV efficiently immortalizes B lymphocytes in vitro to form lymphoblastoid cell lines (LCLs). In established BL cell lines and LCLs, EBV can follow either a latent or productive cycle (reviewed in reference 14).

During latency in LCLs, a subset of the EBV proteins are expressed: EBNAs 1, 2, 3A, 3B, and 3C, EBNA-LP, TP1, TP2, and LMP. The EBNA genes are encoded by related mRNAs formed from a >100-kbp primary transcript containing many alternative splices (11). The EBNAs can be expressed from two promoters, BC-R2 (4) and BW-R1 (27, 31, 36). These operate in a mutually exclusive manner, and during established latency the BC-R2 promoter is used preferentially (45, 46). The BC-R2 and BW-R1 promoters are sometimes called Cp and Wp, respectively. The BC-R2 promoter has several well-characterized control elements; EBNA-1 activates expression through an enhancer within Ori-P (37); glucocorticoids stimulate expression through a glucocorticoid response region called ES-1 (−919 to −1099) (23, 33); EBNA-2 activates expression through a tissue-specific enhancer element at about −430 (27a, 39, 44); and there is an enhancer element at about +450 (42). There is an additional promoter (Fp) controlling the expression of EBNA-1 in BL (30) and nasopharyngeal carcinoma (5) cells. TP1 and LMP are expressed from separate but partly overlapping promoters which are *trans*-activated by EBNA-2 (10, 43, 50). EBNA-2 therefore plays a central role in maintaining the transcription of EBV latency genes and probably affects many cell genes; the control of transcription of EBNA-2 is an important part of the mechanism of latent gene expression of EBV.

Various stimuli can be used to activate EBV from latency in B-lymphocytes; these include tetradecanoyl phorbol acetate (TPA), butyrate, iododeoxyuridine, transforming growth factor-β, cross-linking surface immunoglobulin, superinfection with EBV het virus, or transfection with an expression vector for the EBV gene BZLF1. BZLF1 is one of the first EBV genes expressed during productive cycle activation (35, 40). It is a transcription factor of the b-Zip family and functions via sequence-specific DNA binding to a DNA sequence similar but not identical to the AP-1 site (13, 22). It is active as a homodimer and transactivates its own promoters and the promoters of several EBV early genes. Its expression is organized to amplify any activation signal exceeding a threshold level (15, 35), thus initiating a switch to the cascade of productive cycle gene expression.

Part of the switch may involve BZLF1 with a dual role as both an activator of the productive cycle and a repressor of latency. Cotransfection studies with BZLF1 expression vectors and EBV latency promoter constructs showed that BZLF1 can repress transcription from BC-R2, BW-R1, and the promoter for LMP (21).

Here we investigate the mechanism by which BZLF1 represses transcription from the BC-R2 promoter. We demonstrate that the repression involves interference with the positive signal supplied by the glucocorticoid receptor through ES-1, and we show that this is probably mediated through activation of the cellular proto-oncogene c-*fos*. We also investigated the effects of the synthetic glucocorticoid dexamethasone and of hydrocortisone on latent and productive cycle gene expression in an EBV-positive BL cell line (Jijoye) and an EBV-immortalized human LCL (B95-8CR). There is an inverse coordination of the expression of the latency protein EBNA-2 with the productive cycle proteins BZLF1 and the early antigens.

MATERIALS AND METHODS

Plasmid construction. The eukaryotic expression vectors pCMV19, pCMV-BZLF1, p3.1W, and p3.1W-BZLF1 have been reported elsewhere (28).

The chloramphenicol acetyltransferase (CAT) expression vectors pBLCAT2 and pCA124/123 have been described before (18, 25). To generate p992ΔCAT, the *Kpn*I-*Mbo*I restriction enzyme fragment containing the EBV B95-8

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sequence from 10313 to 11305 (1) was cloned into the *Kpn*I to *Bam*HI restriction enzyme sites of the promoterless vector pUC18CAT (described in reference 35). To generate p791ΔCAT, the *Pst*I-*Mbo*I restriction enzyme fragment containing the EBV B95-8 sequence from 10514 to 11305 was cloned into the *Pst*I to *Bam*HI restriction enzyme sites of pUC18CAT. The plasmid RCAT has been described before (35). p791ΔCAT and p992ΔCAT were made by C. Rooney.

To prepare NZCAT, the *Nae*I restriction enzyme fragment containing EBV sequence 103182 to 104901 was ligated with *Hin*dIII linkers (GCAAGCTTGC). This construction was digested with *Hin*dIII and subcloned into the *Hin*dIII site of pUC18CAT in an orientation so that position 103182 was proximal to the CAT coding sequence.

To make GEM3Z-406BC, the insert from M13 clone 406BC containing EBV sequence from 10793 to 11699 was excised, using the *Bam*HI and *Eco*RI sites in the polylinker. This insert was subcloned into the *Bam*HI and *Eco*RI sites in pGEM3Z (Promega). This plasmid was linearized with *Ava*II, and riboprobes were synthesized from the SP6 promoter, giving a probe containing EBV sequence from 11599 to 10793.

RNA analysis. Cytoplasmic RNA preparation and Northern (RNA) blotting were done as described previously (12). Probes for Northern blotting were prepared from the M13mp8 clones 406. BC (B95-8 EBV positions 10793 to 11699) and 28.JOY (part of the Jijoye EBNA-2 open reading frame). The RNase protection assay was done essentially as described before (35).

Cell culture. Ramos cells were maintained in RPMI 1640 medium containing penicillin, streptomycin, and 10% heat-inactivated fetal calf serum. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and 10% heat-inactivated fetal calf serum. Where indicated, the serum was exchanged for 10% charcoal-stripped heat-inactivated fetal calf serum (7) for the duration of the experiment.

The Ramos cells were transfected by electroporation at 250 V and 960 μF on a Bio-Rad Genepulser as described previously (6), except that for the steroid-depleted assays, prior to electroporation, the cells were transferred to medium containing 10% charcoal-stripped fetal calf serum and then left for 24 h. After the electroporation, the cells were resuspended in the same medium and left for 24 h. Dexamethasone (Sigma) was solubilized in ethanol and added at a final concentration of 10^{-6} M where indicated. The cells were harvested 24 h later. NIH 3T3 cells were transfected by calcium phosphate coprecipitation (17) except that for the steroid-depleted assays, prior to transfection, the cells were transferred to medium containing 10% charcoal-stripped fetal calf serum and then left for 24 h. The precipitate was left on the cells for 15 h and then washed off, and the cells were maintained in medium containing 10% charcoal-stripped fetal calf serum with 10^{-6} M dexamethasone where indicated for a further 24 h. The cells were harvested 24 h later, and extracts were prepared and assayed as described previously (6). Each experiment was performed at least twice.

For protein analysis, Jijoye and B95-8CR cells were maintained in RPMI 1640 medium containing penicillin, streptomycin, and 10% serum. The serum was either fetal calf serum or charcoal-stripped fetal calf serum, and, where indicated, steroid hormones were added (dexamethasone to 10^{-6} M and hydrocortisone to 2×10^{-5} M. For immunofluorescence, the cells were washed with phosphate-buffered saline, air dried on to glass slides, fixed in methanol-acetone (1:2), and then incubated with each antibody for 30 min at room temperature. For Western immunoblotting analysis, the cells were disrupted in gel loading buffer, separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and incubated with the primary antibodies shown in the figure legends. Then the filters with the monoclonal antibodies were incubated with rabbit anti-mouse immunoglobulin antibody, and all were incubated with ^{125}I-labeled protein A.

DNA-binding assays. Gel retardation assays were performed essentially as described previously (26). Labeled double-stranded oligonucleotide (1 ng) was incubated at 37°C for 30 min with either 2 μl of rabbit reticulocyte lysate or 2 μl of rabbit reticulocyte lysate in vitro-translated BZLF1 together with 50 ng of double-stranded unlabeled competitor oligonucleotide, where indicated, in 100 mM KCl–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9)–10% glycerol–0.2 mM EDTA–4 mM dithiothreitol–100 ng of poly(dI-dC) per ml. The resulting complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE (26).

The oligonucleotides used in the assay are double-stranded versions of the following sequences: M, GAAG CACTGACTCATGAAG (described as 292/293 in reference 26); and GRE, AGCTTAAGTTTATTGGGACACAGTGTC CTTACCACAAGGATGG (24).

RESULTS

BZLF1 downregulation of BC-R2 requires glucocorticoids. Glucocorticoids are an important factor in the control of the expression of the BC-R2 promoter (23). Glucocorticoids bind and activate the glucocorticoid receptor. This is a sequence-specific DNA-binding protein that interacts with glucocorticoid response elements (GRE) in the enhancers of responsive genes, thus activating transcription (3, 9). Glucocorticoid-mediated activation of gene expression can be disrupted by the components of the cell transcription factor AP-1 (20, 24, 32, 47). Since BZLF1 has some homology with c-*fos* (13), we investigated whether BZLF1 repression of the BC-R2 promoter is linked to the glucocorticoid induction of BC-R2. Reporter constructs containing the BC-R2 promoter from −992 to +2 (p992ΔCAT) and from −791 to +2 (p791ΔCAT) cloned upstream of the marker CAT gene (Fig. 1A) were introduced into an EBV-negative BL cell line (Ramos) either with an EBV expression vector for BZLF1, p3.1W-BZLF1, or with the corresponding empty vector, p3.1W. The cells were maintained in medium depleted of steroid hormones. After 24 h, the synthetic glucocorticoid hormone dexamethasone was added where indicated, and the cells were incubated for a further 24 h. The resulting CAT activity (Fig. 1B) revealed that p992ΔCAT is induced by dexamethasone, whereas p791ΔCAT is not induced. This further maps the glucocorticoid-responsive element within BC-R2 to the 3′ part of ES-1, consistent with the position of a palindromic GRE motif, AGCACA(TTT)TGTTCT, at position 10408 to 10422. BZLF1 has no effect on p791ΔCAT expression in either the presence or the absence of dexamethasone. Expression from p992ΔCAT was not affected by BZLF1 in the absence of dexamethasone but was strongly repressed by BZLF1 in the presence of dexamethasone.

This demonstrates that the repression of BC-R2 by BZLF1 occurs only when the sequence between −791 and −992 is intact and dexamethasone is present. The results suggest that the repression is mediated by BZLF1 interference with glucocorticoid-induced expression.

BZLF1 represses glucocorticoid-induced gene expression.

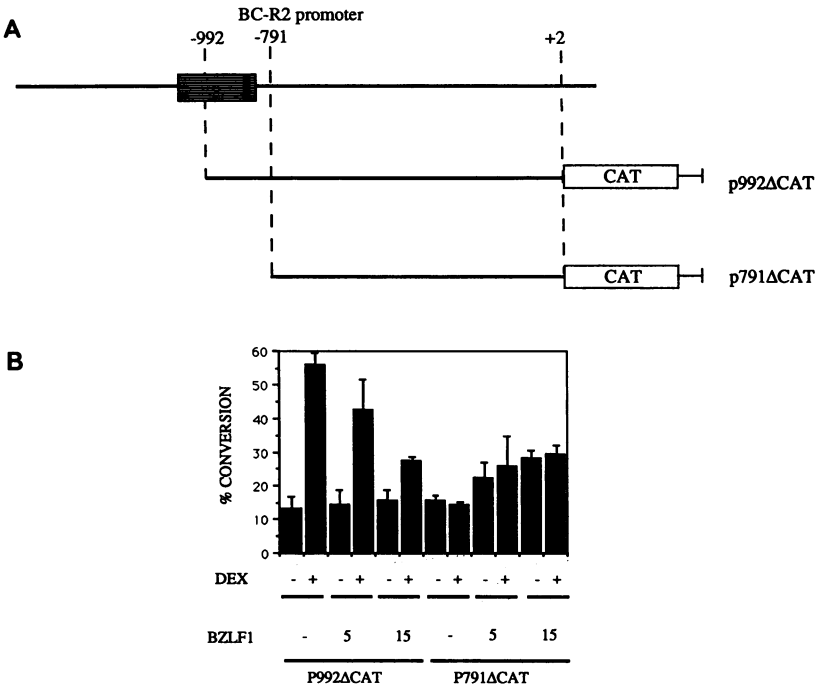

FIG. 1. BZLF1 repression of BC-R2 requires glucocorticoids. (A) Plasmid names are shown to the right of the maps. The CAT coding sequence is represented by an open box, and promoter sequences are shown as broad lines. The numbers refer to the sequence coordinates relative to the transcription initiation point (+1) of each promoter. The striped box represents the position of ES-1, and the solid box within it marks the position of the palindromic GRE sequence. (B) p992ΔCAT or p791ΔCAT was transfected with 0, 5, or 15 μg of p3.1W-BZLF1, balanced to 15 μg with p3.1W, into Ramos cells maintained in 10% charcoal-stripped serum. After 24 h, dexamethasone (DEX) was added where indicated, and 24 h later the cells were harvested and CAT activity was determined. Each bar represents the results from two experiments, and the standard error of the mean (SEM) is indicated.

To define the mechanism of BZLF1 repression of BC-R2 activity, we investigated the ability of BZLF1 to repress transcription through a synthetic GRE. We compared the expression vector pBLCAT2 (Fig. 2A) (25), which contains a herpes simplex virus thymidine kinase promoter upstream of the reporter gene CAT and which is unresponsive to dexamethasone (18), with pCA124/123 (18), which is derived from pBLCAT2 and contains a synthetic GRE cloned in the polylinker upstream of the thymidine kinase promoter (Fig. 2A). pBLCAT2 and pCA124/123 were transfected into NIH 3T3 cells (which respond well to glucocorticoids [32]) with either an expression vector for BZLF1 (pCMV-BZLF1) or the parental vector lacking the BZLF1 sequence (pCMV19). The cells were first maintained in steroid-depleted medium; 24 h later, the synthetic glucocorticoid hormone dexamethasone was added where indicated, and the cells were incubated for a further 24 h. The cytomegalovirus promoter used to control the expression of BZLF1 is not affected by dexamethasone (data not shown). The resulting CAT activity (Fig. 2B) reveals that BZLF1 had a negligible effect on the level of expression either from pBLCAT2 in the presence or absence of dexamethasone or from pCA124/123 in the absence of dexamethasone. However, BZLF1 clearly repressed expression from pCA124/123 in the presence of dexamethasone. The only difference between these two promoters is the presence of the GRE in pCA124/123, so this experiment establishes that BZLF1 can interfere with glucocorticoid receptor-mediated activation of transcription through the GRE.

BZLF1 does not interact with the GRE. BZLF1 is a sequence-specific DNA-binding protein which interacts with defined sequence elements (13, 22). Some sequence-specific DNA-binding proteins are capable of interacting with more than one type of DNA sequence element (34). One possible mechanism for the observed downregulation of glucocorticoid-induced gene expression by BZLF1 could involve a direct interaction of BZLF1 with the GRE, blocking a productive interaction between the glucocorticoid receptor and the GRE. We therefore investigated whether BZLF1 interacts directly with the GRE in vitro. Labeled double-stranded oligonucleotides containing either a BZLF1 binding site (M) or a glucocorticoid receptor binding site (GRE) were used to assay complex formation with BZLF1 (Fig. 3). BZLF1 protein was synthesized in an in vitro translation system and incubated with the probes, and then the free and complexed oligonucleotides were separated by electrophoresis through a 4% polyacrylamide gel. BZLF1 forms a complex with the M oligonucleotide which is competed with specifically by a 50-fold molar excess of unlabeled M but is not affected by a 50-fold molar excess of GRE. In a more sensitive assay, GRE was used as the labeled oligonucleotide, and no complex formation between BZLF1 and the GRE was observed. These experiments show that BZLF1 does not interact with the GRE and suggest that the observed interference of BZLF1 with glucocorticoid-induced gene expression is not mediated through a direct interaction of BZLF1 with the GRE.

BZLF1 repression of glucocorticoid-induced gene expression is indirect. The N-terminal part of BZLF1 has some homology with the region of c-*fos* shown to be involved in interaction with the glucocorticoid receptor (13, 24). This raised the possibility that the repression of glucocorticoid-

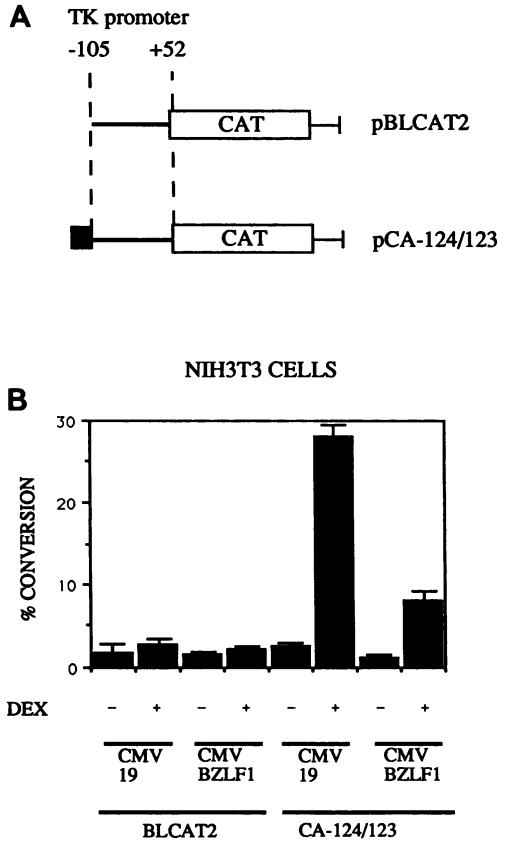

FIG. 2. BZLF1 repression of glucocorticoid-induced gene expression acts through the GRE. (A) Plasmid names are shown to the right of the maps. The CAT coding sequence is represented by an open box, and promoter sequences are shown as broad lines. The numbers refer to the sequence coordinates relative to the transcription initiation point (+1) of each promoter. The stippled box represents the synthetic GRE. TK, thymidine kinase. (B) pBLCAT2 or pCA124/123 was transfected together with pCMV19 or pCMV-BZLF1 into NIH 3T3 cells maintained in 10% charcoal-stripped serum. After 24 h, dexamethasone (DEX) was added where indicated; 24 h after that, the cells were harvested and CAT activity was determined. Each bar represents the results from two experiments, and the SEM is shown.

induced gene expression by BZLF1 was the result of a direct interaction of BZLF1 with the glucocorticoid receptor. In cross-linking experiments, we could detect no in vitro association between BZLF1 and the glucocorticoid receptor (data not shown). We also found no effect of dexamethasone on the ability of BZLF1 to transactivate a responsive gene (data not shown), providing further evidence that BZLF1 and the glucocorticoid receptor do not interact directly.

Transient-transfection experiments with promoter-CAT constructs have shown that BZLF1 can activate a cotransfected human c-*fos* promoter through interaction with three BZLF1 binding sites in the promoter (16). We investigated the ability of BZLF1 to activate the endogenous human c-*fos* gene. p3.1W and p3.1W-BZLF1 were introduced into Ramos cells, and 48 h later the cells were harvested. Western blot analysis with an antibody against c-Fos (Fig. 4) shows that BZLF1 up regulates c-Fos levels in human B cells.

Therefore, the most likely mechanism for BZLF1 repression of the BC-R2 promoter involves BZLF1 transactivation of c-*fos*. The c-Fos protein can then interact with the

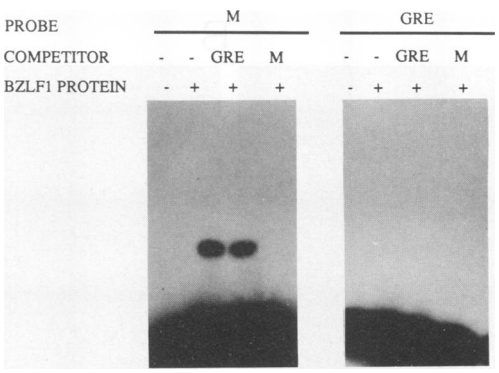

FIG. 3. BZLF1 affinity for probes M and GRE. Rabbit reticulocyte lysates were used to translate either no RNA (−BZLF1) or BZLF1 RNA (+BZLF1). The resulting proteins were incubated with the radioactive GRE and M double-stranded oligonucleotide probes in the presence of a 50-fold molar excess of the unlabeled competitor where indicated. The free and bound oligonucleotide complexes were separated on a 4% polyacrylamide gel.

glucocorticoid receptor (20, 24), interfering with the glucocorticoid receptor-mediated activation of BC-R2.

Steroid hormones are an important determinant of EBV latency. Because of the structure of the EBNA transcription unit and the transactivation function of EBNA-2, any event regulating expression from the BC-R2 promoter would be expected to affect the expression of most other latent genes. Previous reports on the effect of steroids on latency investigated EBV strains Raji (38) and Daudi (2, 8), which have both suffered major deletions within the EBNA transcription unit. To understand better the importance of steroid hormone control of latency, we investigated the effects of steroids on two cell lines containing nondefective EBV, Jijoye (a B-type EBV-positive BL cell line) and B95-8CR (an A-type LCL). The cells were maintained in medium containing different steroid hormones; after 3 days, the proportion of cells in the productive cycle was determined by immunofluorescence with a monoclonal antibody to the EBV pro-

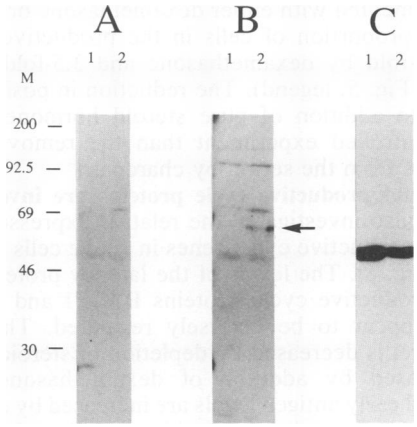

FIG. 4. BZLF1 upregulates c-*fos*. Ramos cells were transfected with 5 μg of p3.1W or p3.1W-BZLF1. After 48 h, the cells were harvested, and c-Fos protein levels were determined by Western blot analysis. (A) Normal rabbit serum, 1:500, (B) rabbit anti-Fos (19) serum, 1:500; (C) β-tubulin antibody, 1:2,500. The positions of the molecular mass standards are shown on the left (in kilodaltons). The arrow by panel B shows the position of c-Fos protein.

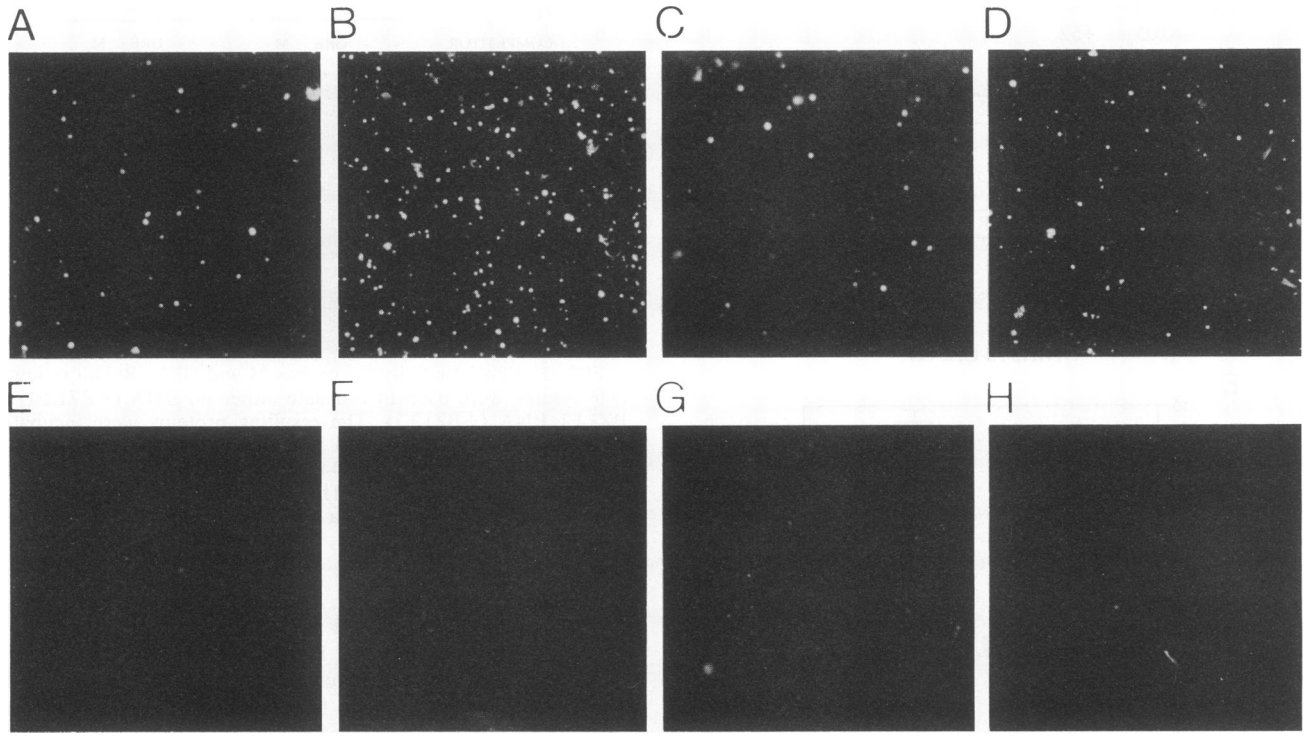

FIG. 5. Steroid hormones alter EBV latency. Jijoye cells were maintained for 3 days in different media: A and E, 10% serum; B and F, 10% charcoal-stripped serum; C and G, 10% charcoal-stripped serum plus dexamethasone; D and H, 10% charcoal-stripped serum plus hydrocortisone. Cells expressing BZLF1 were identified by indirect immunofluorescence with (A to D) BZ1 antibody (49) or (E to H) no antibody. Typical numbers of BZLF1-positive cells per field corresponding to panels A to D were as follows. Jijoye: A, 9; B, 25; C, 6; D, 7. B95-8CR: A, 92; B, 129; C, 32; D, 36.

ductive cycle protein BZLF1 (Fig. 5). A proportion of cells maintained in normal serum are in the productive cycle. For both cell lines, the proportion of cells in the productive cycle was increased when the cells were maintained in medium depleted in steroid hormones, but this effect was clearest with the Jijoye cells, which had fewer BZLF1-positive cells in normal serum than did B95-8CR cells. In both cell lines maintained in medium depleted of serum steroid hormones but supplemented with either dexamethasone or hydrocortisone, the proportion of cells in the productive cycle was reduced 4-fold by dexamethasone and 3.5-fold by hydrocortisone (Fig. 5, legend). The reduction in positive cells in response to addition of pure steroid hormone is a more clearly controlled experiment than the removal of many compounds from the serum by charcoal.

Latent and productive cycle proteins are inversely regulated. We also investigated the relative expression of EBV latent and productive cycle genes in Jijoye cells by Western blotting (Fig. 6). The levels of the latency protein EBNA-2 and the productive cycle proteins BZLF1 and other early antigens appear to be inversely regulated. The EBNA-2 protein level is decreased by depletion of steroid hormones and increased by addition of dexamethasone, whereas BZLF1 and early antigen levels are increased by depletion of steroid hormones and decreased by addition of dexamethasone.

Our rationale for steroid hormone control of latency assumes that EBNA transcription in Jijoye and B95-8CR cells is directed by the BC-R2 promoter, but this has not been fully established. RNA mapping of the C1 exon demonstrated that B95-8CR cells used the BC-R2 promoter (Fig.

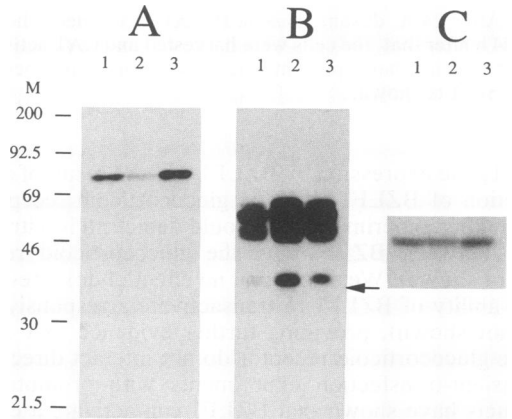

FIG. 6. Inverse regulation of productive cycle and latency proteins by glucocorticoids. Jijoye cells were maintained for 3 days in different media: 1, 10% serum; 2, 10% charcoal-stripped serum; 3, 10% charcoal-stripped serum plus dexamethasone. Extracts of the cells were separated on 10% polyacrylamide–sodium dodecyl sulfate gels and analyzed by Western blotting. (A) PE-2 monoclonal antibody (48) to EBNA-2 (1:5,000, ascites); (B) human serum (EE) recognizing EBV early antigens (1:2,500); (C) monoclonal antibody to β-tubulin (Amersham; 1:5,000). The positions of the molecular mass standards are shown on the left (in kilodaltons). The arrow in panel B shows the position of BZLF1.

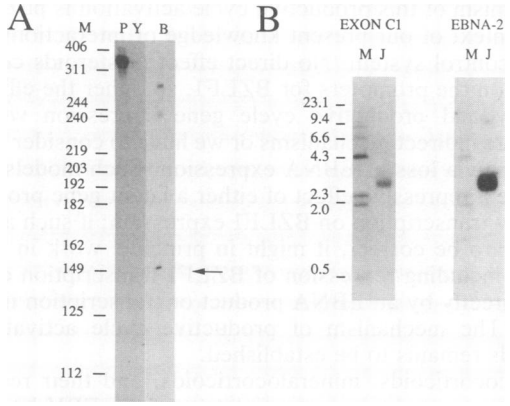

FIG. 7. Structure of EBNA transcripts. (A) Cytoplasmic RNA from B95-8CR cells was hybridized with a riboprobe (GEM3Z-406BC *Ava*II) spanning the C1 exon. The RNase-resistant fragments were separated on a 6% polyacrylamide denaturing gel and visualized by autoradiography. M, molecular size markers (*Msp*I digest of pBR322 end labeled with Klenow DNA polymerase), in nucleotides; P, undigested input probe; Y, yeast RNA; B, B95-8CR RNA. The arrow marks the position of the 148-bp fragment protected by the C1 exon. (B) Polyadenylated cytoplasmic RNA from Jijoye cells was glyoxalated, electrophoresed with DNA size markers, and blotted to nitrocellulose. The filters were hybridized to a probe spanning exon C1 (4O6BC) or the EBNA-2 coding sequence (28.JOY). M, molecular size markers (*Hin*dIII digest of phage lambda DNA), in base pairs; J, Jijoye RNA.

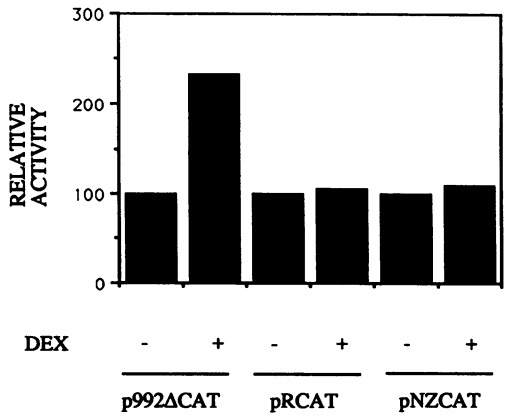

FIG. 8. Effect of glucocorticoids on activity of BZLF1 promoters. Plasmids are shown below each lane. Each plasmid was transfected into Ramos cells maintained in 10% charcoal-stripped serum. After 24 h, dexamethasone (DEX) was added where indicated. The CAT activity in the absence of dexamethasone is set at 100%, and the CAT activity in the presence of dexamethasone is shown relative to this. Each transfection was performed twice, and the SEM was less than 1%.

7A). Jijoye cells also use the BC-R2 promoter (4, 44), but the pattern of mRNA hybridizing to the C1 exon is different from that found in B95-8, and no cDNA clones have been analyzed to confirm the EBNA transcription pattern. We therefore performed Northern blot analysis with C1 exon and EBNA-2 coding sequence probes to establish that both probes detect similar-sized transcripts (Fig. 7B). This is compatible with BC-R2 controlling transcription of EBNA-2 in Jijoye cells. We therefore conclude that both dexamethasone and hydrocortisone promote latency in cell lines that use the BC-R2 promoter and that transcription from BC-R2 includes mRNA for EBNA-2.

The inverse response of latent and productive cycle proteins to glucocorticoids indicates that their control is coordinated. The increase in productive cycle proteins when steroids were depleted (Fig. 5 and 6) was unexpected. We know that transcription from BC-R2 is regulated directly by dexamethasone through interaction of the glucocorticoid receptor with region ES-1. The altered level of EBNA-2 protein observed could correlate directly with altered activity of the BC-R2 promoter. The increase in BZLF1 levels is not thought to play a role in the decrease in EBNA proteins because BZLF1 cannot down-regulate BC-R2 in steroid-depleted medium. Also, the reduction in EBNA-2 levels (Fig. 6) is too great to be accounted for by the proportion of cells which switch to the productive cycle. The inverse coordination of latency and productive cycle gene expression described here suggests that the expression of productive cycle genes may also be controlled by steroids.

BZLF1 promoters do not appear to be regulated by glucocorticoids. Computer analysis of the whole EBV sequence identified three possible GREs (23), but only ES-1 was responsive to steroids in functional assays. It remains possible that there are other control points for transcription of EBV genes by glucocorticoids, such as direct interaction at other nonconsensus GREs or control by glucocorticoid-induced genes. We tested the effect of dexamethasone on the activity of the promoters for the 2.8-kb mRNA encoding both BRLF1 and BZLF1 and the promoter for the 1.0-kb mRNA encoding BZLF1. Transfection studies in Ramos cells with RCAT (promoter for the 2.8-kb mRNA), NZCAT (promoter for the 1.0-kb mRNA), and p992ΔCAT showed that in conditions in which p992ΔCAT is activated by dexamethasone, neither BZLF1 promoter was affected by dexamethasone (Fig. 8).

DISCUSSION

Simple immunofluorescence studies on spontaneously productive cultures of cells containing EBV reveal that individual cells either react strongly with the antibodies to productive cycle proteins or are essentially unreactive (illustrated in Fig. 5). This shows the extreme nature of the switch between latency and the productive cycles; much of our research on virus reactivation has been directed to understanding the molecular mechanisms that constitute the switch. Autoactivation of the promoters for BZLF1 and BRLF1 is part of the mechanism (15, 35), and we show here that BZLF1 not only serves to activate the productive cycle but also simultaneously represses latent cycle gene expression from the BC-R2 promoter. This is achieved through inhibition by BZLF1 of the glucocorticoid receptor-mediated activation of the BC-R2 promoter (Fig. 9). EBV infection generally induces the expression of the glucocorticoid receptor in lymphocytes (41), and this induction might be part of the mechanism of switching from the BW-R1 promoter to BC-R2 which occurs during establishment of the latent infection. Although BZLF1 has some N-terminal protein sequence homology to c-Fos in the approximate part of c-Fos which can inhibit the glucocorticoid receptor by direct binding, we have found no evidence that BZLF1 binds directly to the glucocorticoid receptor. Rather, BZLF1 seems to inhibit the glucocorticoid receptor by causing a prolonged activation of the cellular c-*fos* gene and consequent indirect inhibition of the BC-R2 promoter. This repres-

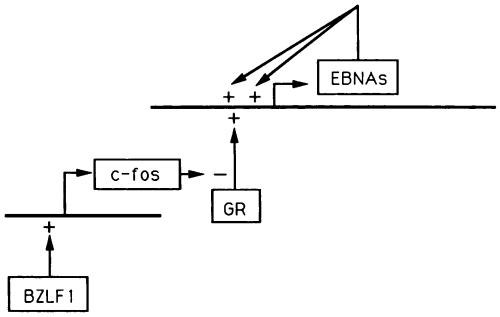

FIG. 9. Summary of interactions involved at BC-R2. Two products from BC-R2, EBNA-1 and EBNA-2, transactivate the BC-R2 promoter. Glucocorticoids activate the BC-R2 promoter through interaction of the glucocorticoid receptor at the GRE within ES-1. BZLF1 inhibits the glucocorticoid-induced expression from BC-R2. This occurs because BZLF1 transactivates the promoter for the cellular gene c-*fos*; the c-Fos protein is known to interact with and inhibit the glucocorticoid receptor (GR).

sion of latent cycle gene expression by BZLF1 further enhances the quality of the switch between latency and the productive cycle. The effect of BZLF1 on c-Fos also raises the possibility that BZLF1 influences the expression of other (cellular) genes by interference with glucocorticoid receptor-induced expression.

The significance of the dependence of the BC-R2 promoter on steroids is not yet clear, and the relative quantitative significance of the activation of BC-R2 from the ori-P enhancer, the GRE, and other promoter elements and downstream enhancers remains to be determined. The dependence of EBNA-2 expression on glucocorticoids demonstrated here (Fig. 6) indicates that the GRE is quantitatively important. The glucocorticoid response was first studied in detail by Kupfer and Summers (23). We have further defined the glucocorticoid-responsive sequence to the region between 10313 and 10514, and it seems very likely to be mediated by the consensus GRE found in this region.

It is presumed that, in vivo, some lymphocytes move from full latent gene expression to the restricted pattern of gene expression, making only EBNA-1. Such cells can at present only be recognized in BL (29), but it is widely hypothesized that such restricted latent gene expression might be characteristic of long-lived B cells infected with EBV, which perhaps evade immune surveillance by failing to express the full (immunogenic) complement of EBNAs. The BC-R2 promoter is not used in these BL cells, and EBNA-1 transcription is initiated at a different promoter, starting transcription at about 62233 on the B95-8 sequence (30). Loss of glucocorticoid receptor activity is one plausible mechanism by which shutdown of the BC-R2 promoter could be achieved in these cells. This would result in reduced levels of EBNA-2 and consequent further reduction in transcription from BC-R2 and the promoters for LMP, TP1, and TP2.

In addition to downregulation of latency protein expression, withdrawal of steroids from the medium also caused activation of the productive cycle, suggesting a further point of control. In those experiments, steroids were removed from the serum by using charcoal, and it is likely that this removes many factors from serum. Nevertheless, productive cycle activation in such stripped serum is prevented by supplementation with dexamethasone, so steroids presumably are the active agent being removed by the charcoal. The mechanism of this productive cycle activation is puzzling in the context of our present knowledge of interactions in the EBV control system. No direct effect of steroids could be found on the promoters for BZLF1, so either the effects on latency and productive cycle gene expression work by separate indirect mechanisms or we have to consider models working via loss of EBNA expression. Such models would require a repressive effect of either a latent gene product or latency transcription on BZLF1 expression; if such a model proves to be correct, it might in principle work in several ways, including repression of BZLF1 transcription directly or indirectly by an EBNA product or transcription interference. The mechanism of productive cycle activation by steroids remains to be established.

Glucocorticoids, mineralocorticoids, and their receptors thus emerge as important regulators of the EBV latent and productive cycles. This may be important both in the context of the established complex effects of steroids on B lymphocytes and in possible virus reactivation in vivo in response to treatment with steroid antagonists, for example, the abortifacient RU486.

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