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Promiscuous *trans* Activation of Gene Expression by an Epstein-Barr Virus-Encoded Early Nuclear Protein

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We identified an Epstein-Barr virus (EBV) gene product which functions in transient-expression assays as a nonspecific *trans* activator. In Vero cells, cotransfection of the *Bgl*II J DNA fragment of EBV together with recombinant constructs containing the bacterial chloramphenicol acetyltransferase (CAT) gene gave up to a 100-fold increased expression of CAT activity over that in cells transfected with the recombinant CAT constructs alone. The *Bgl*II J fragment acted promiscuously, in that increased CAT synthesis was observed regardless of whether the promoter sequences driving the CAT gene were of EBV, simian virus 40, adenovirus, or herpes simplex virus origin. Cleavage of cloned *Bgl*II-J plasmid DNA before transfection revealed that activation was dependent upon the presence of an intact BMLF1 open reading frame. This was confirmed with subclones of *Bgl*II-J and with hybrid promoter-open reading frame constructs. This region of the genome is also present in the rearranged P3HR-1-defective DNA species, and defective DNA clones containing these sequences produced a similar activation of CAT expression in cotransfection experiments. The heterogeneous 45–60-kilodalton polypeptide product of BMLF1 may play an important regulatory role in expression of lytic-cycle proteins in EBV-infected lymphocytes.

Infection of B lymphocytes with Epstein-Barr virus (EBV) leads to the establishment of permanent lymphoblastoid cell lines in which the virus is maintained predominantly in the latent state. Multiple copies of the EBV genome are present as circular episomes, and expression of viral products is limited to certain Epstein-Barr nuclear antigens (EBNAs), the latency membrane antigens, and two small RNA polymerase III transcripts (12, 22–24, 36, 50). A small proportion of the cells in permissive lines show spontaneous activation of lytic-cycle products, and expression of these early antigen and virus capsid antigen polypeptides can also be induced by treatment with chemical agents (e.g., sodium butyrate or phorbol esters) or by superinfection with the EBV P3HR-1 virus isolate. However, the response to these treatments is both variable and limited. The absence of an efficient tissue culture system for lytic infection and the lack of virus mutants have limited the experimental approaches available for identifying and studying the EBV regulatory genes involved in expression of early and late lytic-cycle polypeptides.

In cells productively infected with herpes simplex virus (HSV), regulated expression of the virus genome results in the sequential appearance of the immediate-early (IE), delayed-early, and late groups of viral gene products. Functional IE polypeptides are required for delayed-early protein synthesis (26), and analysis of temperature-sensitive mutants in the IE175 (ICP4) protein revealed that at nonpermissive temperatures delayed-early mRNA synthesis is blocked (45, 46). The dominant control of expression of the different polypeptide classes apparently occurs at the level of initiation of transcription, and heterologous genes linked to IE promoters are regulated as IE genes (25, 44). A direct role for the IE175 and IE110 (ICP0) proteins in the stimulation of transcription of delayed-early genes was recently obtained by using transient-expression systems and cloned IE175 and IE110 genes (14, 16, 41, 42, 47). In adenovirus infection, synthesis of the E1A protein increases synthesis of messenger RNA for the E2, E3, and E4 regions (3, 30, 38), and transcription of simian virus 40 (SV40) late genes is activated (independently of DNA synthesis) by the SV40 T antigen (5, 6, 31, 32).

Seeking to identify functionally equivalent EBV regulatory proteins, we used a system in which expression of a readily assayable gene, that for chloramphenicol acetyltransferase (CAT), was examined in cells cotransfected with a variety of EBV DNA fragments. The CAT gene has been widely used and has been found to provide a sensitive and valid assay for promoter activity (18). For example, HSV infection of cells transfected with HSV-CAT constructs stimulates expression of these recombinant genes in a pattern which correctly reflects the characteristics (IE or delayed early) of the parental viral genes (40). By this approach, we identified an EBV product, the MS-EA polypeptide, an early antigen encoded by the *Bam*HI-M-S open reading frame, which functions as an efficient *trans* activator of gene expression both in cotransfection assays and when expressed in a permanent fibroblast cell line.

MATERIALS AND METHODS

Cells and transfections. Vero cells were cultured at 37°C in Dulbecco modified Eagle minimal essential medium (DMEM) containing 10% fetal calf serum. BEMS-8837 and BE55-9 cells (obtained from M.-S. Cho) were grown in DMEM containing 10% fetal calf serum and 1% tylocene. Both cell lines are G418-resistant baby hamster kidney (BHK) cells that have been cotransfected with pSV2neo and plasmids containing defective EBV sequences. BEMS-8837, as reported previously (10), was transfected with pTS6 DNA and shows positive immunofluorescence for the MS-EA protein. BE55-9 was transfected with pMC9, a plasmid containing the *Pst*I repeat region of the defective genome, and served as a control in transfection and immunofluorescence experiments. Transfections were performed by procedures described by O'Hare and Hayward (40). Cells were

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plated the day before into six-well cluster dishes (Costar, Cambridge, Mass.) at 4×10^5 cells per well. Between 4 and 5 h before transfection, the medium was replaced with 2.5 ml of fresh DMEM containing 10% fetal calf serum and antibiotics (penicillin and streptomycin). DNA to be transfected was added to 125 μl of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid)-buffered saline containing 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 12 mM glucose, and 42 mM HEPES (pH 7.03). A 12-μl portion of 2.5 M $CaCl_2$ in enough water to bring the total transfection cocktail up to 250 μl was added dropwise to the HEPES-DNA over a 30-s period with continuous bubbling. Precipitates were allowed to form for 25 min at room temperature, and the mixture was then added dropwise to the cell culture medium. Between 4 and 5 h after transfection, the medium was removed and cells were treated with 1 ml of 15% glycerol in serum-free DMEM for 1 min. Cells were then washed with phosphate-buffered saline and incubated at 37°C in 2.5 ml of fresh DMEM with 10% fetal calf serum. For MS-EA induction, BEMS cells were treated with DMEM containing 10% fetal calf serum and 3 mM sodium butyrate (*n*-butyric acid-sodium salt; Sigma Chemical Co., St. Louis, Mo.) immediately after glycerol treatment. Butyrate-containing medium was aspirated and replaced by fresh medium after 12 h.

Plasmids. The following EBV DNA recombinant plasmids were cloned in pBR322 unless otherwise stated. Plasmids pSL93 and pSL77 contain the *Bam*HI C fragment and *Bam*HI H fragments from B95-8 (20), pGD4 contains the *Sal*I F fragment of B95-8, and pDH33 contains the *Bam*HI BG fragment from P3HR-1. The plasmids pM-B2-C, pM-B2-J, and pM-B2-K contain the *Bgl*II C, *Bgl*II J, and *Bgl*II K fragments of M-ABA cloned in pHC79 (43) and were obtained from G. Bornkamm, University of Freiburg, Freiburg, West Germany. Plasmid pPL9 is a *Hin*dIII-*Bgl*II subclone of pM-B2-J. Plasmid pPL10 was constructed by ligating the 2,150-base pair (bp) *Hin*dIII-*Bam*HI fragment of *Bam*HI-M into *Bam*HI-*Hin*dIII-cleaved pTJ278 DNA, thus placing the open reading frame, termed the *Bam*HI-M leftward reading frame 1 (BMLF1), under the control of the IE promoter of simian cytomegalovirus. In plasmid pPL12, the complete MS-EA coding region (BMLF1 plus BSLF2) was placed under the control of the IE promoter of simian cytomegalovirus. The 2,460-bp *Nhe*I-*Xba*I fragment from pM-B2-J was cloned into *Xba*I-cleaved pKP58 vector DNA by Keith Peden. The EBV sequences were subsequently moved as a *Bgl*II-*Hin*dIII fragment into *Bam*HI-*Hin*dIII-cleaved pTJ278 DNA to form pPL12. The plasmids pTS6, pTS5, pTS1, pMC9, and pMC14 are all subclones of the P3HR-1-defective DNA fragment *Bam*HI-W'C' (9). Plasmids pMC14, pTS6, pTS5, and pTS1 contain the MS-EA gene, and their construction was described previously (10). Plasmid pMC9 consists of a 6.2-kilobase *Eco*RI-*Bam*HI fragment of *Bam*HI-W'C' inserted between the *Eco*RI and *Bam*HI sites of pSV2neo. The EBV sequences contain the *Pst*I repeat gene (DS_R) plus the 75-bp repeats located between the *Bam*HI-M rightward reading frame 2 (BMRF2) and BMLF1 in *Bam*HI-M.

The CAT gene was placed under the control of two different EBV promoter/regulatory regions. In pDH123 the CAT gene was expressed from the *Not*I repeat gene promoter and contains sequences from +46 to −1031 of the *Not*I gene. A 1,180-bp *Hin*cII-partial *Nae*I subfragment of *Bam*HI-H was cloned into pBR322 after the addition of *Hin*dIII and *Bam*HI linkers to form pPL3. The *Hin*dIII-*Bam*HI fragment of pPL3 containing the EBV sequences was then ligated into *Hin*dIII-*Bam*HI-cleaved pCATB' to give pDH123. In pMC31, the CAT gene is expressed from the promoter for the EBV MS-EA polypeptide (10). An 810-bp *Bam*HI-*Spl*I subfragment of *Bam*HI-S (pSL95) containing sequences from +76 to −730 of the MS-EA gene was inserted upstream of the CAT gene in pCATB' to form pMC31. Other CAT constructs used included TK-CAT (pPOH3), in which the CAT gene is expressed from the promoter for the HSV thymidine kinase gene (41); pCATB' and pKCAT23 (both obtained from O. Andrisani and N. Jones, Purdue University, West Lafayette, Ind.), which contain, respectively, the CAT structural gene without any eucaryotic promoter region and the CAT gene fused to the adenovirus type 5 E3 control region (E3-CAT) (54); pSV2-CAT and pA10-CAT (obtained from G. Khoury, National Institutes of Health, Bethesda, Md.), in which the CAT gene is controlled by the early regulatory region of SV40 either with the 72-bp enhancer sequences (pSV2-CAT) or without the enhancer region (pA10-CAT) (34); and pGH47, in which the palindromic sequences upstream from the EBV *Not*I promoter were inserted into pA10-CAT. In pGH47, the upstream region from −11 to −1031 of the EBV *Not*I gene was fused to the minimal SV40 early promoter that drives CAT expression. This was achieved by taking the 1,020-bp *Hin*cII-*Nae*I fragment from EBV *Bam*HI-H (pSL77), adding *Hin*dIII and *Bam*HI linkers to the respective termini, and recloning in pBR322 as pPL4. The *Bgl*II-*Bam*HI fragment of pA10-CAT containing the minimal SV40 early promoter and the CAT gene was then ligated into the *Bam*HI site of pPL4 to form pGH47.

The plasmid pIGA15 contains the HSV IE110 gene (16) and was a gift from I. Gelman and S. Silverstein, Columbia University, New York, N.Y.

CAT assays. Cell harvesting and CAT assays were performed essentially as described by Gorman et al. (18) and O'Hare and Hayward (40, 41). At approximately 48 h after transfection, cells were washed with phosphate-buffered saline and then scraped into 1 ml of 0.04 M Tris hydrochloride (pH 7.4)–1 mM EDTA–0.15 M NaCl. The cells were pelleted and stored at −20°C or assayed directly. Pellets were suspended in 100 μl of 0.25 M Tris hydrochloride (pH 7.8), sonicated for 5 s, and centrifuged for 5 min at 10,000 rpm and 4°C in a rotor (SS34; Ivan Sorvall, Inc., Norwalk, Conn.). A 100-μl portion of standard reaction mixture containing 70 μl of 1 M Tris hydrochloride (pH 7.8), 7 μl of H_2O, 2 μl of 40 mM acetyl coenzyme A, and 0.2 μCi of [^{14}C]chloramphenicol (40 to 50 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was added to each supernatant and incubated for 1 h at 37°C. Chloramphenicol and its acetylated products were extracted with 1 ml of ethyl acetate and evaporated in a speed-vacuum centrifuge. Dried samples were suspended in 20 μl of ethyl acetate, spotted on glass-backed, silica gel, thin-layer chromatography plates, and resolved by ascending chromatography in chloroform-methanol (95:5) solvent. Plates were exposed against Kodak XR-2 film. Percent acetylations were determined by scraping the silica gel off the glass plates into toluene-PPO (2,5 diphenyloxazole)-POPOP (1,4-bis-[5'phenyloxazoly]benzene) scintillation fluid and counted in an LKB 1216 liquid scintillation counter (LKB Instruments, Inc., Rockville, Md.). Chloramphenicol-3-acetate, chloramphenicol-1-acetate, and chloramphenicol 1,3-diacetate were counted as a single acetylated product in percent acetylation determinations.

Samples of a high-expression plasmid, pSV2-CAT, and a promoterless plasmid, pCATB', were included in each set of

experiments to define the range of CAT activity in each experiment. All experiments were performed on multiple occasions and with independent DNA preparations. In two experiments, the stability of the transfected DNA was assayed by determining the plasmid copy number at the time of harvesting (1). No difference in the copy number of the target DNA was detected between samples cotransfected with *Bgl*II-J and those cotransfected with carrier pBR322 DNA. In general, because of variations in transfection efficiencies, comparisons between experiments should be considered primarily qualitative.

Immunofluorescence. Cells were cultured on Lab-Tek tissue culture slides (4 chamber; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) in DMEM containing 10% fetal calf serum. At 1 day after plating, the culture medium was made 3 mM in sodium butyrate and, after treatment for 12 h, the butyrate-containing medium was replaced by fresh medium. At 48 h after initiation of butyrate treatment, the slides received three 5-min rinses in cold Tris-saline (20 mM Tris hydrochloride, [pH 7.4], 12.5 mM $CaCl_2$, 10 mM $MgCl_2$, 150 mM NaCl) and the cells were fixed in ice-cold acetone for 5 min. After air drying, slides were either stored at −20°C or rehydrated in Tris-saline for 5 min. A 10-μl portion of a 1:40 dilution of MS-EA-positive human serum was applied to each chamber and incubated for 45 min at 37°C. After rinsing in Tris-saline, the cells were incubated with a 1:40 dilution of fluorescein isothiocyanate-linked goat anti-human immunoglobulin G (IgG) (Cooper Biomedical, Inc., West Chester, Pa.) for 30 min followed by a final rinse in Tris-saline.

RESULTS

Identification of an EBV DNA fragment which functions as a *trans* activator. Synthesis of the early and late antigens characteristic of the viral lytic cycle can be induced by treatment of EBV-infected lymphoblastoid cells with chemical agents such as sodium butyrate or phorbol esters or by superinfection with P3HR-1 virus carrying rearranged defective DNA species. To identify *trans*-acting viral products which might have a role in switching on lytic-cycle genes, we used a readily assayable target gene, the bacterial CAT gene, in a transient-expression system. The CAT gene was placed under the control of the promoter region for an early EBV transcript, the *Not*I repeat transcript arising from BHLF1 in *Bam*HI-H (29). This *Not*I-repeat-promoter construct (NRP-CAT) in plasmid pDH123 was transfected into Vero cells alone or in combination with a variety of other cloned EBV DNA fragments, and the amount of CAT enzyme in the Vero cells was assayed 2 days after transfection. Compared with the expression of the CAT gene from the complete SV40 early promoter in pSV2-CAT, the level of basal CAT expression from the NRP-CAT construct was very low (Fig. 1). Cotransfection of NRP-CAT with a cloned fragment of P3HR-1-defective DNA (pMC9) or with the standard genome fragments *Bam*HI-C, *Bam*HI-H, *Bgl*II-C, *Bgl*II-K, or *Sal*I-F produced no significant alteration in the levels of CAT activity. (The salient features of each of these clones are listed in Table 1). In contrast, extracts from Vero cells which received the NRP-CAT construct together with *Bgl*II-J showed a dramatic 60-fold increase in activity over cells receiving NRP-CAT alone. A positive (10-fold) effect was also observed on cotransfection with the 16.2-kb fragment *Bam*HI-BG (Fig. 1). In this report, we focused on the *Bgl*II-J activity.

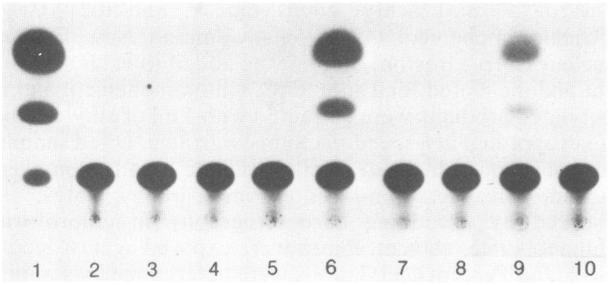

FIG. 1. Identification of an EBV gene product capable of *trans* activating CAT gene expression. Vero cells were transfected with 1.5 μg of a control plasmid, pSV2-CAT (lane 1), or the hybrid EBV *Not*I-repeat-promoter-CAT construct, pDH123, either alone (lane 10) or together with 1.5 μg of a variety of different cloned EBV DNA fragments (lanes 2 to 9). Cells were harvested 48 h after transfection, and equal amounts of extracts were assayed for CAT activity. The cotransfected EBV plasmids were the defective DNA clone pMC9 (lane 2), *Bam*HI-H (lane 3), *Bgl*II-C (lane 4), *Bgl*II-K (lane 5), *Bgl*II-J (lane 6), *Bam*HI-C (lane 7), *Sal*I-F (lane 8), and *Bam*HI-BG (lane 9).

TABLE 1. Salient features of the clones used

Clone	EBV sequence(s)	Salient feature(s)[a]
pSL93	*Bam*HI-C	ori-P; EBERs
pSL77	*Bam*HI-H	*Not*I gene
pM-B2-K	*Bgl*II-K	*Pst*I gene
pM-B2-C	*Bgl*II-C	EBNA-2 ORF
pGD4	*Sal*I-F	EBNA-1 ORF
pMC9	Defective DNA	*Pst*I gene; 75-bp repeats
pDH33	*Bam*HI-BG	16.2 kilobases
pM-B2-J	*Bgl*II-J	MS-EA
pPL9	Subclone *Bgl*II-J	MS-EA
pPL10	BMLF1	Fused to SCMV promoter
pPL12	BMLF1 and BSLF2	Fused to SCMV promoter
pMC14	Defective DNA	MS-EA
pTS6	Defective DNA	MS-EA
pDH123	1,080 bp of *Bam*HI-H	NRP-CAT
pGH47	1,020 bp of *Bam*HI-H	NRE-A10-CAT
pMC31	810 bp of *Bam*HI-S	MS-EA-CAT

[a] ori-P, Origin of EBV plasmid replication; EBERs, Epstein-Barr early RNAs; EBNA, Epstein-Barr nuclear antigen; ORF, open reading frame; SCMV, simian cytomegalovirus; NRP, *Not*I repeat promoter; NRE, *Not*I repeat enhancer.

***Bgl*II-J is a promiscuous activator of gene expression.** The *Bgl*II J fragment activated CAT synthesis from an EBV promoter/regulatory region (Fig. 1). To determine whether *Bgl*II-J activation was specific for EBV promoters or represented a more generalized *trans*-activating mechanism, cotransfection experiments were undertaken with *Bgl*II-J and hybrid constructs in which the CAT gene was linked to promoter regions from other viruses, namely: (i) the early region of SV40 that retained the 72-bp enhancer sequences (pSV2-CAT) or lacked the enhancer sequences (pA10-CAT) or in which these enhancers were replaced by the palindromic region upstream of the EBV *Not*I repeat promoter (pGH47); (ii) the HSV thymidine kinase gene (TK-CAT); and (iii) the E3 gene of adenovirus type 5 (E3-CAT). *Bgl*II-J proved to be promiscuous in its ability to *trans* activate. For example, CAT expression from the TK-CAT, E3-CAT, and pGH47 hybrid constructs was increased 50-, 60-, and 80-fold, respectively, by cotransfection with *Bgl*II-J (Fig. 2). Even pSV2-CAT, which gives very high basal levels of CAT expression, could be further stimulated by *Bgl*II-J cotransfection (sevenfold increase in expression over pSV2-CAT alone in this experiment).

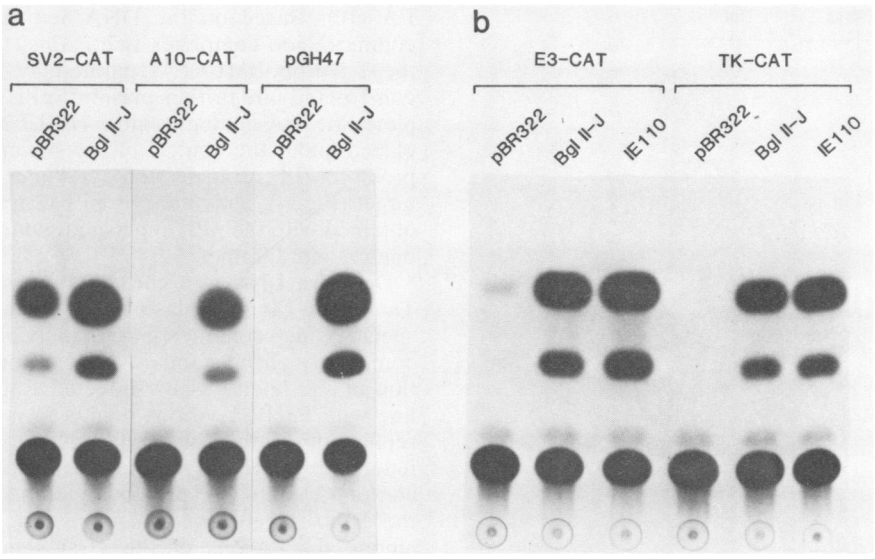

FIG. 2. Lack of target promoter specificity for *trans* activation by *Bgl*II-J. Vero cells were cotransfected with 1.5 μg of (a) pBR322 or *Bgl*II-J and 1.5 μg of plasmid DNAs containing the CAT gene fused to different forms of the SV40 early promoter, namely, the complete promoter (SV2-CAT), the minimal promoter (A10-CAT), or a substituted minimal promoter (pGH47); and (b) pBR322, *Bgl*II-J, or HSV IE110 plasmid DNAs and target hybrid plasmids containing the CAT gene driven by the adenovirus E3 promoter (E3-CAT) or the HSV thymidine kinase promoter (TK-CAT). At 48 h after transfection, equal amounts of extracts were assayed for CAT activity.

In transient-expression assays, the HSV IE110 (ICP0) gene specifically activates expression of only the delayed-early classes of HSV genes when the HSV IE174 (ICP4) gene product is also present (41, 42). However, in the absence of the IE174 product, IE110 acts as a nonspecific *trans* activator (P. O'Hare, J. P. Mosca, and G. S. Hayward, *in* T. Grodzicker et al., ed., *Cancer Cells*: vol. 4, DNA tumor viruses: control of gene expression and replication, in press). We also compared *Bgl*II-J and HSV IE110 cotransfections with the E3-CAT and TK-CAT targets (Fig. 2). Both *Bgl*II-J and HSV IE110 activated these targets, and in each case the fold induction given by *Bgl*II-J was comparable to that given by HSV IE110. This observation also held when HSV IE110 and *Bgl*II-J were cotransfected with targets such as the HSV-derived 175K-CAT and 38K-CAT constructs and the EBV-derived NRP-CAT construct (data not shown). *Bgl*II-J and HSV IE110 thus have in common the ability to efficiently *trans* activate heterologous targets.

An intact BMLF1 is required for biological activity. The *Bgl*II J fragment contains all, or portions of, three open reading frames (2; Fig. 3): an incomplete BMRF2; a complete BMLF1, together with its upstream promoter region; and an intact BSLF1 minus its promoter region. To identify the biologically active region within *Bgl*II-J, cotransfection experiments were performed with *Bgl*II-J which had been cleaved before transfection with restriction enzymes which cut once within the EBV sequences. Cleavage of the EBV sequences away from the vector sequences with *Bgl*II did not significantly alter *Bgl*II-J activation of CAT genes in the pGH47 or E3-CAT constructs (Fig. 3). Similarly, the *trans*-activation properties were not diminished by cleavage with *Bst*EII or *Nru*I, both of which are single-cut enzymes cleaving within BSLF1. On the other hand, cleavage with *Nco*I or *Eco*RI drastically reduced *Bgl*II-J activation of CAT expression from cotransfected plasmids. The two inactivating enzymes cleaved within BMLF1, suggesting that the product of this reading frame is required for the activation effect.

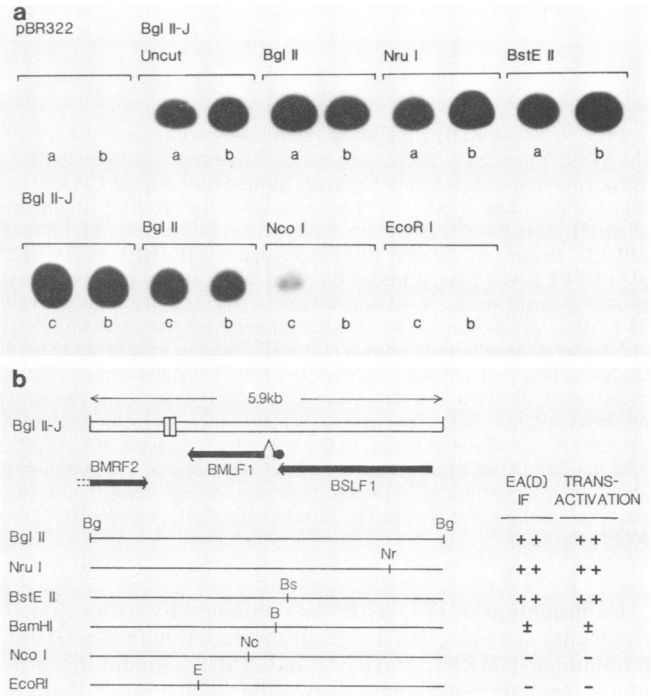

FIG. 3. *trans* Activation is dependent upon expression of a *Bgl*II-J gene product. (a) Target CAT plasmid pGH47 (a), pE3-CAT (b), or pA10-CAT (c) was cotransfected with pBR322 DNA, *Bgl*II-J, or *Bgl*II-J which had been cleaved with the indicated restriction enzymes. CAT activity was assayed in transfected cell extracts after 48 h, and the relative amounts of chloramphenicol-3-acetate product are presented. (b) Diagrammatic representation of the structure of *Bgl*II-J showing the location of the open reading frames and the 72-bp repeats (□) as reported by Baer et al. (2). The effect of cleavage on *trans*-activating ability is correlated with the effect on synthesis of EBV early antigen, EA(D) as measured by an indirect immunofluorescence assay using human serum.

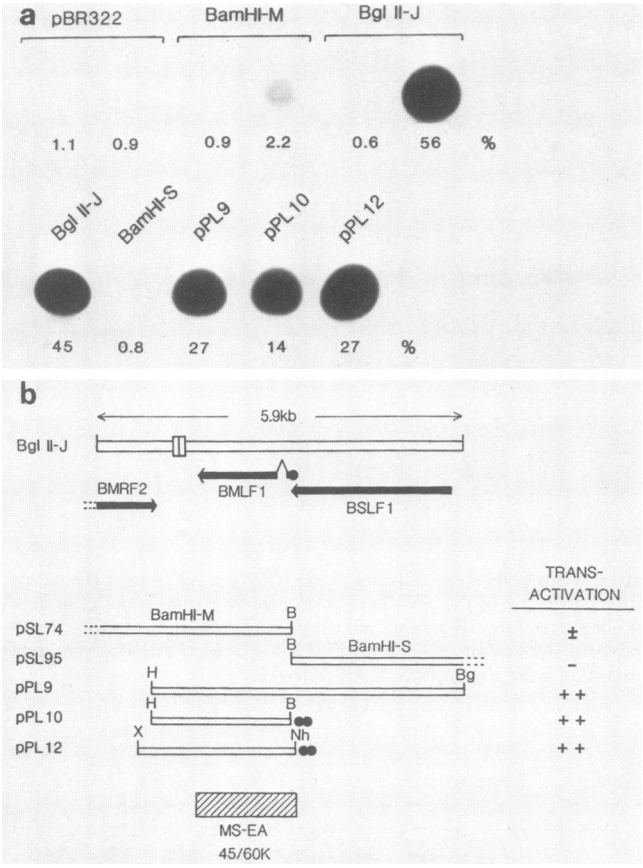

FIG. 4. *trans*-Activating ability correlates with the presence of the MS-EA gene. To further define the active region within *Bgl*II-J, cotransfection experiments were performed with target CAT plasmids and either cloned DNAs spanning the *Bgl*II-J fragment (*Bam*HI-M or *Bam*HI-S) or subclones of *Bgl*II-J (pPL9, pPL10, and pPL12). In the pPL10 and pPL12 constructs, BMLF1 or BMLF1 plus BSLF2 was placed under the control of a strong constitutive promoter from simian cytomegalovirus. CAT activity was assayed 48 h after transfection. The relative amounts of the chloramphenicol-3-acetate product are shown (autoradiographic spots) along with the percentage of substrate acetylated (%). Cotransfection experiments were performed with a variety of target plasmids. (a) Results of cotransfection with, in pairs, pCATB′ (which lacks a eucaryotic promoter) and pTK-CAT and, in the lower half, with pA10-CAT. (b) The diagrammatic representation of the structure of the subclones shows the correlation between *trans*-activating ability and the presence of the MS-EA gene. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Nh, *Nhe*I; X, *Xba*I.

Confirmation of this result was obtained by using cloned fragments spanning *Bgl*II-J. Cotransfection with *Bam*HI-M (containing BMRF1, BMRF2, and BMLF1 minus its promoter region) gave only a twofold activation, whereas *Bam*HI-S (containing BSLF1 and 90% of BSLF2) had no effect on CAT expression (Fig. 4). A subclone of *Bgl*II-J lacking BMRF2 (pPL9) also gave efficient *trans* activation, further indicating that activation by the product of BMLF1 is not dependent on the presence of either the BMRF2 or BSLF1 product. Indeed, BMLF1 alone, when placed behind a strong heterologous promoter (that for the IE gene of simian cytomegalovirus) in the construct pPL10 gave *trans* activation in cotransfection experiments (Fig. 4). We previously characterized the product of this region of the genome as a 45–60-kilodalton nuclear early protein we termed MS-EA (10). Based on the DNA sequence (2), the MS-EA-coding region comprises two exons, the 60-bp BSLF2 and the 1,274-bp BMLF1, separated by a 169-bp intron. We constructed one further plasmid, pPL12, in which the complete MS-EA-coding region (BSLF2 plus BMLF1) was placed under the control of the simian cytomegalovirus IE promoter. In cotransfection experiments with A10-CAT as a target (Fig. 4), activation by pPL12 was comparable to that obtained with the pPL10 plasmid, which lacks the first small open reading frame.

Defective DNA also encodes the *trans*-activating protein. The P3HR-1 lymphoblastoid cell line carries defective DNA species which contain approximately one-third of the genetic complexity of the standard EBV genome (8, 9). Superinfection of the latently EBV-infected Raji lymphoblastoid cell line with P3HR-1 virions results in induction of lytic EBV replication in the Raji cells. Lytic-cycle proteins are not induced by P3HR-1 preparations lacking the defective population (37, 48). We previously cloned and mapped one of the P3HR-1-defective DNA species (9) and showed that among the regions of the viral genome retained in this defective species was the MS-EA gene (10). Two defective DNA clones containing this region were tested for *trans*-activating function by cotransfection with pA10-CAT into Vero cells. Plasmids pMC14 and pTS6 both activated CAT expression from the minimal SV40 early promoter in pA10-CAT (Fig. 5). The construct pTS5 differs from pTS6 in that a deletion introduced into pTS5 removes the 75-bp repeats, the polyadenylation signal for the MS-EA mRNA transcript, and approximately 50 bp from the 3′ terminus of BMLF1. In pTS5-transfected Vero cells, a trace amount of MS-EA polypeptide can be detected by immunoblot analysis and a proportion of the cells (0.1% versus 4 to 5% for pTS6) stain for MS-EA by indirect immunofluorescence (10). As might be expected, given the limited expression of MS-EA from pTS5, cotransfection of pTS5 gave only a twofold activation of CAT synthesis (25-fold less than that seen with pTS6).

Features of MS-EA *trans* activation. In cotransfected Vero cells, the extent of *trans* activation of the target CAT gene was dependent on the amount of MS-EA gene in the transfection mixture. Increasing the amount of pTS6 led to increased expression from 1.5 μg of the cotransfected pGH47 plasmid, with a 7.5-fold activation at 0.25 μg of pTS6 and a 116-fold activation at 3 μg of pTS6 (Fig. 6a). Cells cotransfected with equivalent amounts of the deleted MS-EA variant, pTS5, showed a 20-fold lower level of CAT expression than that seen with the intact MS-EA.

That MS-EA can efficiently *trans* activate its own expression is illustrated in Fig. 6b, in which CAT expression is measured in cells transfected with a construct, pMC31, in which the CAT gene was fused to the MS-EA promoter. Vero cells transfected with pMC31 alone showed very limited CAT expression. However, expression increased 70-fold on cotransfection with *Bgl*II-J.

***trans* Activation in an established MS-EA cell line.** The experiments presented in the preceding sections demonstrated that MS-EA can activate expression of target genes in cotransfection assays. We further sought to demonstrate *trans* activation by MS-EA expressed from pTS6 plasmid DNA integrated into the genome of an established cell line. The BEMS-8837 cell line was described previously (10) and was established by cotransfecting BHK cells with pSV2neo and pTS6 DNAs and selecting G418-resistant colonies expressing MS-EA. The resultant BEMS cell lines showed little constitutive synthesis of MS-EA (0.5% positive by indirect immunofluorescence and undetectable by im-

munoblot analysis), but MS-EA expression was inducible by treatment of the cell lines with 3 mM sodium butyrate. In the BEMS-8837 line, 10% of the cell population showed brilliant nuclear staining after sodium butyrate induction (Fig. 7b). The experimental protocol involved transfecting target CAT DNAs into BEMS cells which were then either subjected or not subjected to a 12-h butyrate treatment to induce MS-EA expression. A potential complication arises from the fact that butyrate treatment can itself alter transfection efficiency and expression from transfected plasmids in a nonspecific manner (17). To differentiate between changes in expression resulting from the nonspecific butyrate effect and changes brought about by the induction of MS-EA, an equal amount of each target-DNA cocktail was transfected in parallel into a control BHK cell line (lacking the MS-EA gene but comparable to the extent that it too had been G418 selected after transfection with pSV2neo and an EBV-defective DNA plasmid). The basal level of CAT expression from four transfected CAT constructs was very similar in the BEMS and control cells (Fig. 7, A and B, respectively, open bars). Butyrate treatment of the control pMC31-, TK-CAT-, and E3-CAT-transfected cells resulted in a three- to fourfold increase in CAT expression, and this is taken as a measurement of the nonspecific (background) effect of butyrate treatment. CAT enzyme in equal amounts of extract from butyrate-treated BEMS cells showed greater activation than that of the control cells, a 10-fold increase for pMC31 and E3-CAT and a 20-fold increase for TK-CAT. We interpret this increase over the background butyrate effect to indicate that the induced MS-EA in the BEMS cells was *trans*-activating expression from these input CAT targets.

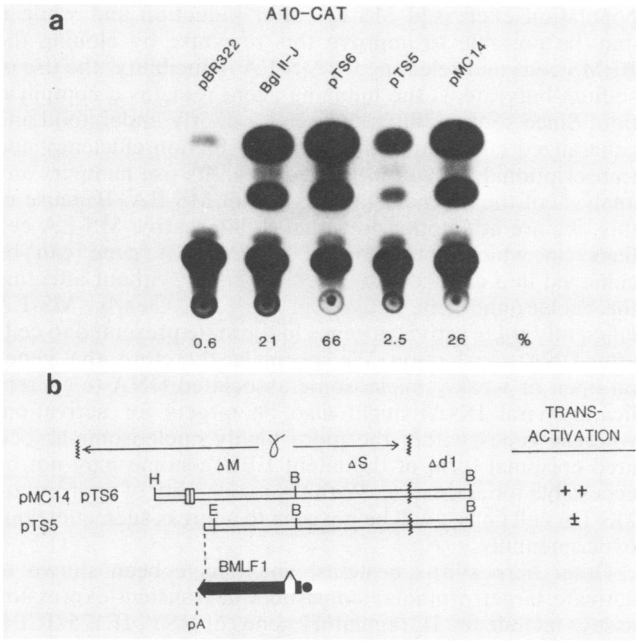

FIG. 5. Defective DNA encodes the MS-EA *trans* activator. (a) Vero cells were cotransfected with 1.5 mg of A10-CAT and 1.5 μg of the MS-EA gene originating from either wild-type DNA (*Bgl*II-J) or P3HR-1-defective DNA (pTS6, pTS5, and pMC14). The plasmid pTS5 differs from pTS6 by the introduction of a 3′ deletion into the MS-EA gene. Equal amounts of cell extract were assayed for CAT activity. The percentage of substrate acetylated is indicated (%). (b) Diagrammatic representation of the structure of the P3HR-1-defective DNA clones, showing the location of the MS-EA gene. The defective DNA sequences map predominantly to the γ segment of the defective genome (5, 7). Δ, Only a portion of the standard fragment is present; ⋛, novel fusion junctions present in defective DNA; B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI.

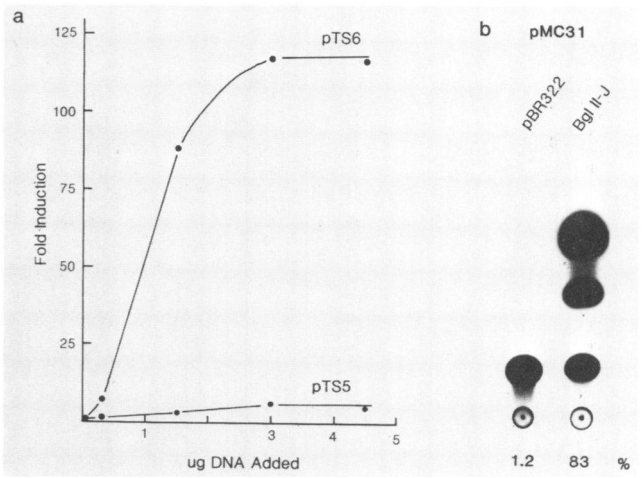

FIG. 6. (a) Response of the CAT target gene to increasing amounts of *trans* activator. Plasmid pGH47 (1.5 μg) was cotransfected into Vero cells with the indicated amount of plasmid DNA carrying the intact (pTS6) or deleted (pTS5) MS-EA gene and extracts were assayed for CAT activity. (b) The MS-EA protein *trans* activates its own expression. The CAT gene under the control of the MS-EA promoter (pMC31) was cotransfected into Vero cells with either pBR322 DNA or *Bgl*II-J DNA, and extracts were assayed for CAT activity. The percentage of substrate acetylated is indicated (%).

DISCUSSION

We have shown that EBV encodes a protein referred to as MS-EA, which functioned in transient DNA assays as a promiscuous *trans* activator of gene expression. MS-EA activated not only EBV promoters but also those of other, unrelated viruses. Targets in which the CAT gene was under the control of the promoter region for the HSV thymidine kinase gene or the E3 gene of adenovirus or driven by the minimal SV40 early promoter were efficiently activated by MS-EA. In contrast to the adenovirus E1A protein, which represses transcription from the complete SV40 early promoter that retains the 72-bp repeat elements (4, 53), expression from the complete SV40 promoter was increased by MS-EA (see Fig. 2a).

The MS-EA promoter appears to function only weakly in fibroblast cells in the absence of other EBV gene products. The amount of CAT enzyme detected in Vero cells transfected with a hybrid construct in which the CAT-coding sequences were fused to the MS-EA promoter region was comparable to that expressed from the minimal SV40 early promoter in pA10-CAT. However, CAT expression from the MS-EA construct reached levels quantitatively similar to those seen with pSV2-CAT when the MS-EA-CAT plasmid was cotransfected with *Bgl*II-J. Thus MS-EA showed positive autoregulation. The ability of MS-EA to stimulate its own expression explains our previous observation that, compared with other EBV gene products, the MS-EA protein is readily detectable both by immunofluorescence and by immunoblotting in DNA fragment-transfected fibroblast cells (10). The DNA sequence of the intact MS-EA gene within *Bgl*II-J predicts a spliced mRNA with a small (60 bp) 5′ exon, BSLF2, and a large 3′ exon, BMLF1 (2). Recent

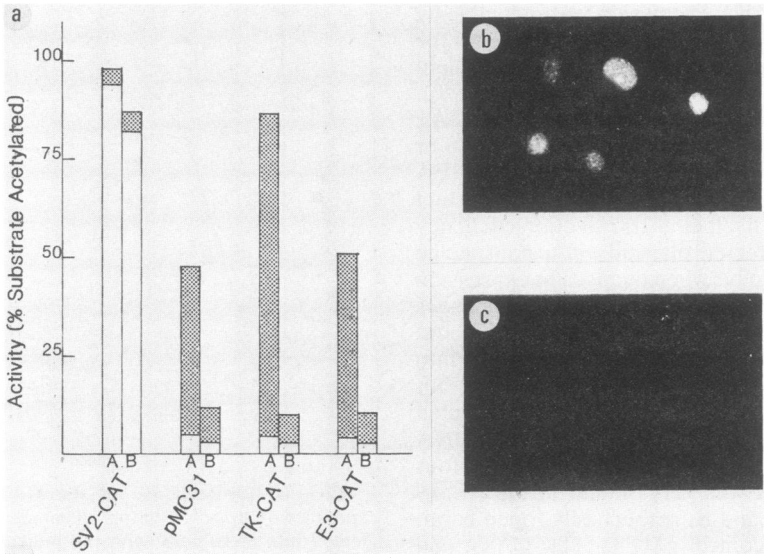

FIG. 7. The MS-EA protein also *trans* activates when expressed in an established cell line. (a) The indicated CAT targets (1.5 μg) were transfected into BEMS-8837 fibroblast cells (A) either with (▨) or without (□) treatment for 12 h with 3 mM sodium butyrate to induce expression of MS-EA or control fibroblast cells (B) either with (▨) or without (□) butyrate treatment. In each case, cell extracts were assayed for CAT activity 36 h after removal of the sodium butyrate. SV2-CAT was included as a standard. (b) and (c) MS-EA expression in BEMS-8837 (b) and control (c) cells after butyrate induction. Cells were stained with EBV-positive human serum and fluorescein isothiocyanate-conjugated anti-human immunoglobulin G in an indirect immunofluorescence assay and were photographed using a 40× lens objective.

data from Sample et al. (49) show that the MS-EA gene is transcribed as a 2.0-kilobase mRNA, which occurs in both spliced and unspliced form in productively infected lymphocytes. We used hybrid promoter-open reading frame constructs to examine whether both open reading frames were required to produce a functional *trans* activator. When driven by the strong constitutive promoter from the IE94 gene of simian cytomegalovirus, the product of BMLF1 alone proved to *trans* activate target genes almost as efficiently as did an equivalent construct containing both open reading frames. Thus it appears that BSLF2 is not essential for this activity. Interestingly, placing the MS-EA-coding region behind a strong constitutive promoter resulted in no greater activation in cotransfection experiments than that achieved by MS-EA driven by its natural promoter, suggesting that the ability of MS-EA to activate its own expression fully compensates for the relative weakness of its promoter.

The EBV (P3HR-1) isolate generates novel defective DNA molecules which consist of four normally noncontiguous regions of the EBV genome (8, 9). Superinfection of latently infected Raji cells by P3HR-1 virus results in a lytic response, with production of early and late viral antigens, and this response is dependent on the presence of defective virus in the P3HR-1 stocks. Clonal isolates of P3HR-1 free of defective DNA do not disrupt latency (48). A defective DNA clone containing the *Bam*HI-Z region activates early antigen and viral capsid antigen synthesis when transfected into D98-HR-1 hybrid cells (11). The MS-EA gene is also encoded by the defective species, and in view of the ability of MS-EA to activate expression of other genes, it seems likely that it too plays some role in the defective DNA-dependent induction of lytic-cycle proteins.

We were able to demonstrate that *trans* activation by MS-EA is not limited to cotransfection assays but also occurs on induction of MS-EA expression from an integrated MS-EA gene in an established cell line. The BEMS-8837 cell line, while valuable, has limitations for studying MS-EA *trans* activation. In the present lines, only 10% of the cell population expressed MS-EA after induction and while it may be possible to improve this response by cloning the BEMS cells and selecting for MS-EA inducibility, the use of sodium butyrate as the inducing agent remains a complication. Since sodium butyrate exerts a poorly understood and generalized effect on both DNA transfection efficiency and transcriptional activity in treated cells, its use hampers any analysis of the mechanism of action of MS-EA. Because of this, we are attempting to establish alternative MS-EA cell lines, in which activation of the MS-EA gene can be achieved in a defined and specific manner without affecting the background gene activity in the cells. Clearly, MS-EA efficiently *trans* activates genes in the state presented to cells after DNA transfection. We speculate, therefore, that genes on open or weakly nucleosome-associated DNA (e.g., replicating viral DNA) might also be targets for activation, whereas genes within the more highly nucleosome-associated episomal form of the latent EBV genome may not be accessible for activation. With the availability of appropriate MS-EA cell lines it will be possible to address such questions experimentally.

Other herpesvirus proteins which have been shown to activate target promoter constructs in transient-expression assays include the IE regulatory genes of HSV, IE175 (ICP4) and IE110 (ICPO) (14, 16, 41, 42, 47), and of pseudorabies virus and the late virion factor of HSV, VP65 (Vmw65) (7, 42). The HSV virion factor and IE175 gave 20-fold greater activation of their natural target genes than of other promoter constructs, whereas IE110 stimulated all heterologous viral or cellular targets tested equally as well as it stimulated homologous HSV targets (O'Hare et al., in press). The pseudorabies virus IE regulatory gene also activates heterologous viral and cellular genes (1, 19, 28), although the degree of activation relative to that with pseudorabies virus targets has yet to be evaluated.

Adenovirus E1A and SV40 large T antigen represent

regulatory proteins from other DNA viruses which, in addition to stimulating their homologous targets (adenovirus E1b, E2, E3, and E4 and the SV40 late gene, respectively), are capable of activating the expression of some heterologous targets (1, 15, 19, 39, 51, 52). In the case of adenovirus E1A, stimulation of transcription of certain cellular genes is observed (15, 19, 39, 51, 52), whereas other heterologous promoter constructs are not activated (1). All these viral *trans*-acting proteins were shown to function by activating transcription from the target promoters. Inducibility by E1A requires the presence of 5′ sequences which appear to overlap with, or be a subset of, those sequences required for optimal basal transcriptional activity (13, 19, 21, 27, 33, 35). Such observations are compatible with the stimulation of transcription involving an increased or more efficient utilization of cellular transcription factor (27, 32, 35).

Since each of the other viral proteins known to have *trans*-activating properties performs key regulatory roles during infection, we suggest that the EBV MS-EA also functions as a regulatory protein in the lytic phase of EBV infection. However, we have yet to determine the mechanism whereby the EBV MS-EA exerts its *trans*-activating effect.

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