MOLECULAR AND CELLULAR BIOLOGY, Nov. 1986, p. 3838–3846
0270-7306/86/113838-09$02.00/0

Vol. 6, No. 11

trans Activation of an Epstein-Barr Viral Transcriptional Enhancer by the Epstein-Barr Viral Nuclear Antigen 1

DAVID REISMAN AND BILL SUGDEN†*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received 18 April 1986/Accepted 30 June 1986

Two regions of the Epstein-Barr virus (EBV) genome together make up an element, *oriP*, which acts in *cis* to support plasmid replication in cells that express the EBV nuclear antigen 1 (EBNA-1). The two components of *oriP* are a region containing a 65-base-pair (bp) dyad symmetry and a region containing 20 copies of a 30-bp direct repeat. Here we show that the 30-bp family of repeats of *oriP* can function as a transcriptional enhancer that is activated in *trans* by the EBNA-1 gene product. In either EBV-genome-positive cells or in cells that express EBNA-1, the 30-bp family of repeats, when positioned in either orientation upstream or downstream, enhances expression of the chloramphenicol acetyltransferase (CAT) gene expressed from either the simian virus 40 early promoter or the herpes simplex virus type 1 thymidine kinase promoter. The extent of transcriptional enhancement varies with the promoter and cell type. This enhanced CAT expression reflects an increased level of CAT mRNA and does not result from amplification of the plasmids expressing CAT. In addition, plasmids carrying the gene for resistance to hygromycin B and the 30-bp family of repeats yielded 10 to 100 times more hygromycin B-resistant colonies than the vector lacking the 30-bp family of repeats in both EBV-genome-positive cells and cells that express EBNA-1. EBNA-1 is known to bind to sequences within the 30-bp family of repeats (D. R. Rawlins, G. Milman, S. D. Hayward, and G. S. Hayward, Cell 42:859–868, 1985), and these *trans*- and *cis*-acting elements together have at least two functional roles: (i) they are required for DNA replication dependent upon *oriP*, and (ii) they can enhance expression of genes linked to the 30-bp family of repeats of *oriP*.

Epstein-Barr virus (EBV) is a human herpesvirus that infects B lymphocytes and transforms them into cells capable of indefinite proliferation in culture (for a recent review, see reference 31). B cells that have been transformed by EBV usually express nuclear antigens known as EBNAs and contain multiple copies of the EBV genome (35, 41). In general, the viral genome is maintained as a supercoiled DNA plasmid of approximately 172,000 base pairs (bp) (2, 27). Three regions of the viral genome are known to be transcribed into poly(A)$^+$ mRNA in transformed cells (1, 45). One region codes for a 62,000-dalton membrane protein (11), and the two others code for the nuclear antigens EBNA-1 and -2 (17, 18, 43). Little is known about the regulation of expression of these genes, and apart from the role of EBNA-1 in EBV plasmid replication (29, 48), little is known about the role of the other expressed EBV genes in B-cell transformation.

A *cis*-acting element, *oriP*, isolated from the EBV genome, allows the replication and maintenance of recombinant plasmids in cells that express EBNA-1 (29, 47, 48). Structural analysis of *oriP* has identified two noncontiguous components that are both required for plasmid replication in the presence of EBNA-1 (20, 36): One component consists of a 20-member family of 30-bp direct repeats, and the other required component, approximately 1,000 bp away in the viral genome, contains a 65-bp dyad symmetry element. Although both components are required in *cis*, their activity is not strictly dependent on their relative distance or orientation (36).

The 30-bp family of repeats has been found to have an additional activity. When this component is inserted into a plasmid carrying a drug resistance marker and the construction is introduced into EBNA-1-positive cells, there is a transient increase in the number of drug-resistant colonies (36). In addition, the 30-bp family of repeats, when present in *cis*, enhances the expression of the chloramphenicol acetyltransferase (CAT) gene approximately 25-fold from the simian virus 40 (SV40) early promoter in the EBV-genome-positive cell line Raji (36). On the basis of these and other observations, we have proposed that EBNA-1 interacts either directly or indirectly with the 30-bp repeats (36). Rawlins et al. (34) have now shown that the EBNA-1 protein protects from digestion with nucleases in vitro both the 30-bp repeats and related sequences in the region containing the dyad symmetry of *oriP*.

To characterize further the properties of this transcriptional enhancer element, we measured its activity in different cell types and its effect on RNA synthesis from two promoters, the SV40 early promoter and the herpes simplex virus type 1 thymidine kinase (HSV-1 TK) promoter. We determined that the *oriP* family of repeats enhances expression from both the SV40 and the HSV-1 TK promoters. The extent of enhancement varies with the promoter, the cells, and the assays used. Enhancement is only found in cells that express EBNA-1. These results indicate that the 30-bp repeats of *oriP* can act as a transcriptional enhancer that is activated in *trans* by the EBNA-1 protein.

MATERIALS AND METHODS

Cell lines. BJAB, ManCa, Ramos, and Wilson are EBV-genome-negative B lymphoblasts derived from EBV-negative Burkitt lymphoma biopsies (24, 25). 721 is an in vitro-transformed EBV-positive B-lymphoid cell line (22). Raji is a EBV-genome-positive cell line obtained from a Burkitt lymphoma biopsy (33). D98/Raji is a somatic cell hybrid between the human epithelial cell line D98 and Raji

* Corresponding author.
† Present address: Department of Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel.

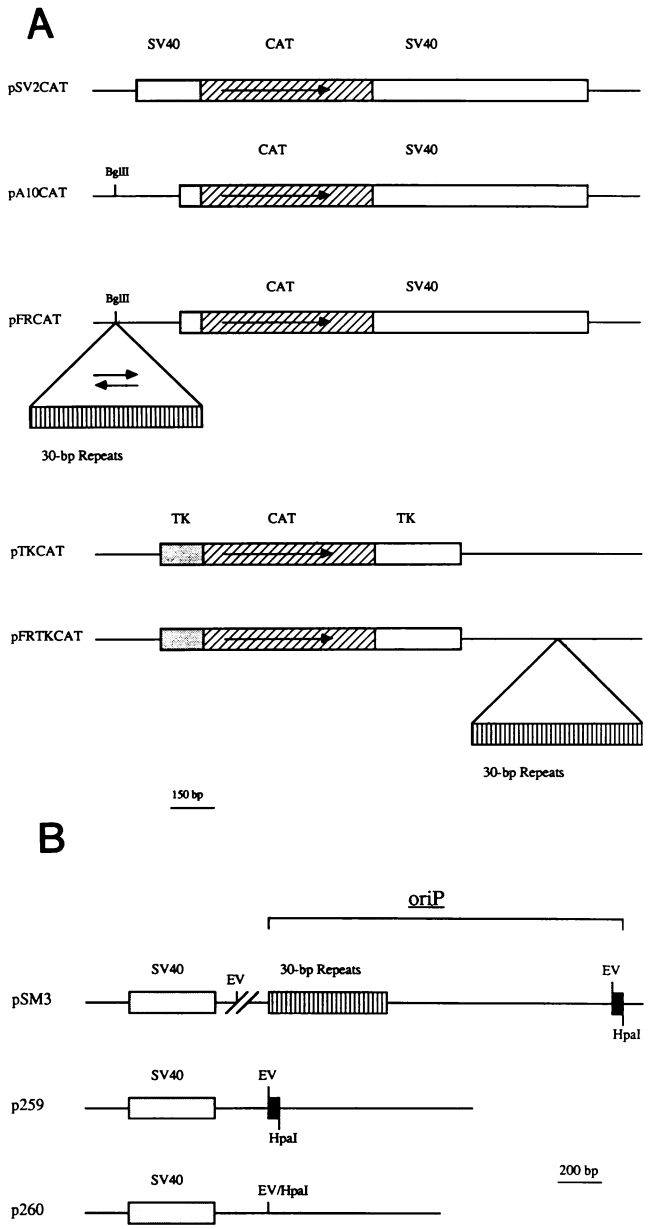

FIG. 1. (A) The 20-member family of 30-bp repeats of *oriP* (box with vertical lines) which is contained between the *Sma*I and *Eco*RI sites at nucleotides 7315 and 8191 on the B95-8 EBV map was ligated to *Bam*HI synthetic linkers. This region of *oriP* was cloned in both orientations (indicated by arrows) into the unique *Bgl*II site of the CAT expression vector $pA_{10}CAT_2$ to generate pFRCAT. $pA_{10}CAT_2$ contains the CAT gene (box with angled lines) downstream of the SV40 early promoter (stippled box) lacking the SV40 72-bp transcriptional enhancer (26). The SV40 early region termination and polyadenylation signals are downstream of the CAT gene (open box). Arrows indicate the direction of transcription. The plasmid pSV_2CAT contains the CAT gene downstream of the SV40 early promoter and includes the 72-bp SV40 transcriptional enhancer (14). pTKCAT and pFRTKCAT were constructed by J. Yates. pTKCAT contains the CAT gene (box with angled lines) linked to the HSV-1 TK gene promoter (stippled box) and HSV-1 TK gene termination and polyadenylation signals (open box). pFRTKCAT contains a 30-bp family of repeats (box with vertical lines) inserted approximately 2,000 bp downstream at the *Eco*RI restriction site of pBR322. (B) pSM3 was constructed by S. Metzenberg. It is derived from pHEBo (41) and contains between the unique *Cla*I and *Hin*dIII restriction sites the *Hpa*II-to-*Hin*dIII fragment of a derivative of SV40 (designated pX-8) containing an insertional mutation in the SV40 origin of DNA replication (stippled box [12]). In pSM3 the SV40 72-bp transcriptional enhancer (within stippled box) and the 30-bp family of repeats of *oriP* (box with vertical lines) are approximately 2,500 and 3,000 bp away, respectively, from the TK promoter. p259 was derived from pSM3 by digestion with *Eco*RV and the deletion of the *Eco*RV-to-*Eco*RV fragment containing the 30-bp family of repeats. In p259 the SV40 enhancer is positioned approximately 400 bp away from the dyad symmetry element (black box) of *oriP*. p260 was derived from pSM3 digestion with *Eco*RV plus *Hpa*I resulting in the deletion of *oriP*.

and contains multiple copies of the EBV genome (13, 44). Human 143 cells were derived from an osteosarcoma (15). 143/EBNA cells express EBNA-1 and were described previously (48). D98/p154 cells carry multiple copies of an *oriP* plasmid that expresses EBNA-1 and were described previously (48). Wilson/p205 cells are derivatives of Wilson cells that carry multiple copies of *oriP* plasmids that express EBNA-1. The plasmid p205 expresses a deleted form of EBNA-1 that lacks a repetitive glycine and alanine sequence of approximately 230 amino acids and has been described previously (48). Wilson/p274.4 cells are derivatives of Wilson that carry one or more integrated copies of the EBNA-1 gene expressed from the SV40 early promoter.

All the B-lymphoid cells plus D98/Raji were grown in RPMI culture medium containing 10% fetal bovine serum. Wilson cells that carry plasmids with *oriP* were grown in the presence of 300 μg of hygromycin B per ml. D98 and 143 and derivatives of these cells carrying *oriP* plasmids were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. D98/p154 cells were grown in the presence of 150 μg of hygromycin B per ml.

Recombinant plasmids. The plasmids used in this study are depicted in Fig. 1 and were constructed as follows. The 20-member family of 30-bp repeats of *oriP* which is contained between the *Sma*I and *Eco*RI sites at nucleotides 7315 and 8191 on the EBV map (B95-8 strain) was ligated to *Bam*HI synthetic linkers. This region of *oriP* was cloned in both orientations into the unique *Bgl*II site of the CAT expression vector $pA_{10}CAT_2$ to generate pFRCAT (26). The plasmid pSV_2CAT contains the CAT gene downstream of the SV40 early promoter and includes the 72-bp SV40 transcriptional enhancer (14).

The two required components of *oriP* (36), the 30-bp family of repeats and a region containing a 65-bp dyad symmetry, were cloned into the *Bam*HI site of pHyg to generate p2.8 and p3.2, respectively. pHyg expresses resistance to the drug hygromycin B from the HSV-1 TK promoter and has been described previously (41). pHEBo is a derivative of pHyg that contains an intact copy of *oriP* (41).

pSM3 was constructed by S. Metzenberg. It is derived from pHEBo and contains between the unique *Cla*I and *Hin*dIII restriction sites of pHEBo the *Hpa*II-to-*Hin*dIII fragment of a derivative of SV40 containing an insertional mutation in the SV40 origin of DNA replication (12). In pSM3 the SV40 72-bp transcriptional enhancer and the 30-bp family of repeats of *oriP* are approximately 2,500 and 3,000 bp away from the HSV-1 TK promoter, respectively (Fig. 1B). p259 was derived from pSM3 by digestion with *Eco*RV to delete the *Eco*RV-to-*Eco*RV fragment containing the 30-bp family of repeats. In p259 the SV40 enhancer is positioned approximately 400 bp away from the dyad symmetry element of *oriP* (Fig. 1B). p260 was derived from pSM3 by digestion with *Eco*RV plus *Hpa*I resulting in the deletion of *oriP* (Fig. 1B).

TABLE 1. CAT expression from recombinant plasmids in EBV-genome-positive and -genome-negative cell lines[a]

Cell line	EBV genome	Avg % chloramphenicol acetylated from extracts of cells carrying plasmids:						
		$pA_{10}CAT_2$	pSV_2CAT	pFRCAT	Ratio pFRCAT/$pA_{10}CAT_2$	pTKCAT	pFRTKCAT	Ratio pFRTKCAT/pTKCAT
A. CAT expression from SV40 early promoter								
Raji	+	0.14	8	5	35 (4)			
Raji	+	0.1	5	1.5[b]	15 (1)			
721	+	0.5	16	5	10 (2)			
D98/HR-1	+	0.1	0.5	5	50 (1)			
Wilson	−	0.1	11	0.1	1 (3)			
Ramos	−	0.1	1	0.1	1 (1)			
BJAB	−	0.2	5	0.1	0.5 (1)			
ManCa	−	0.1	19	0.1	1 (1)			
B. CAT expression from HSV-1 TK promoter								
Raji	+					0.15	10	66 (2)
721	+					0.1	4.5	45 (1)
Wilson	−					0.1	0.1	1 (1)

[a] All cells except D98/HR-1 were electroporated with 5 μg of each of the recombinant plasmids listed above. D98/HR-1 was transfected with 10 μg of plasmid DNA by calcium phosphate coprecipitation. The cells were harvested after 72 h. Extracts were prepared and incubated with [^{14}C]chloramphenicol (2×10^5 cpm) and 0.53 mM acetyl coenzyme A in 0.25 M Tris hydrochloride (pH 7.8) at 37°C for 60 min. The products were separated by thin-layer chromatography, removed from the thin-layer plate, and counted in a scintillation counter. The CAT activity is expressed as the percentage of the total input chloramphenicol that was acetylated. Background disintegrations per minute were approximately 100 to 600 cpm or 0.05 to 0.3% acetylation. The extent of enhancement by the 30-bp family of repeats (pFRCAT) is expressed as the ratio of the average percent acetylation from extracts of cells carrying pFRCAT to that from extracts of cells carrying $pA_{10}CAT_2$. The number in parentheses represents the number of determinations, and the values presented are the average of those determinations.

[b] The plasmid used in this experiment contained the 30-bp family of repeats in an inverted orientation.

Transfections. DNA transfections were performed by the calcium phosphate coprecipitation method of Graham and van der Eb (16). For CAT assays, adherent cells at 50 to 75% confluence in 100-mm tissue culture dishes (approximately 2×10^6 to 4×10^6 cells) were transfected with 1 ml of a calcium phosphate-DNA precipitate containing 10 μg of plasmid DNA for 30 min at room temperature. After this time, 9 ml of complete medium was added, and the cells were incubated for 6 to 8 h at 37°C and then exposed to 20% glycerol for 2 min and washed. Cells were harvested after 72 h. For lymphoid cells, 5 μg of plasmid DNA was introduced into 2×10^7 exponentially growing cells suspended in 0.5 ml of phosphate-buffered saline by electroporation (a peak current of 3 A with a decay time of approximately 20 ms) (41). After electroporation, the cells were suspended in complete medium to approximately 4×10^5 cells per ml. After 72 h the cells were counted and harvested.

CAT assays. CAT assays were performed as described by Gorman et al. (14) 72 h after transfection. Each assay contained extracts from 10^7 lymphoid cells or 200 μg of protein (28) from adherent cells. The extracts were usually assayed for CAT activity at 37°C for 60 min. The reaction consisted of 150 μl containing 0.25 M Tris hydrochloride (pH 7.8), 0.53 mM acetyl coenzyme A, 0.1 μCi (2×10^5 dpm) of [^{14}C]chloramphenicol (47.8 mCi/mmol), and 50 μl of cell extract. The products were separated by ascending thin-layer chromatography and quantitated by scintillation counting. Control experiments indicated that the assay was linear with respect to time for at least 60 min with extracts that yielded 35% acetylated chloramphenicol. Acetylation of 1% was equivalent to the conversion of approximately 21 pmol of chloramphenicol to its acetylated derivatives.

DNA replication assay. The DNA replication assay has been described previously (36). Briefly, plasmid DNAs (5 μg) were introduced into 5×10^7 Raji cells by electroporation. After 96 h, extracts containing low-molecular-weight DNA were prepared by the method of Hirt (19) and digested with the restriction endonuclease *Dpn*I. The products were separated on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled vector DNA. The autoradiograms were scanned by laser densitometry.

Selection of cells resistant to hygromycin B. After plasmid DNAs were introduced into cells by either $CaPO_4$-mediated transfection or electroporation, the cells were propagated for 2 to 3 days without selection. The cells were then assayed for their viability and diluted in 10-fold decrements. Adherent cells were plated in 60-mm dishes starting with 1×10^5 to 2×10^5 cells per dish. Nonadherent cells were plated at 10^6 cells per 90-mm dish (three dishes) and then at 1.5×10^5, 1.5×10^4, 1.5×10^3, and 1.5×10^2 cells per well in six wells each of a 24-well culture dish. Hygromycin B was added immediately to the nonadherent cells and to the adherent cells 24 h after plating. The cells were fed with fresh medium containing hygromycin B every 5 to 7 days. Nonadherent cells plated in 90-mm dishes were collected after 2 weeks, viable cells were separated from dead ones in a density gradient, and the viable cells were replated in 2 ml of medium to maximize their chance of survival. Populations of viable cells were counted 6 to 7 weeks after the addition of hygromycin B.

Preparation of RNA. Total cellular RNA was prepared by guanidium thiocyanate extraction as described by Chirgwin et al. (7). Poly(A)$^+$ RNA was selected by hybridization to polyuridylic acid-diazothiophenyl paper (Hybond-mAP; Amersham Corp., Arlington Heights, Ill.) in buffer containing 0.5 M NaCl, 10 mM Tris hydrochloride (pH 7.4), and 1 mM EDTA. The paper was washed extensively in the hybridization buffer, and poly(A)$^+$ RNA was eluted in buffer containing 10 mM Tris hydrochloride (pH 7.4) and 1 mM EDTA.

RNA dot blots. Poly(A)$^+$ RNA was denatured in 7.4% formaldehyde by heating at 60°C for 15 min as described by White and Bancroft (46). Twofold dilutions were prepared and applied to nitrocellulose. The filters were baked at 80°C for 2 h, prehybridized at 68°C for 18 h with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4])–2× Denhardt (8) solution–0.2% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 7.0)–5 mM EDTA–200 μg of dena-

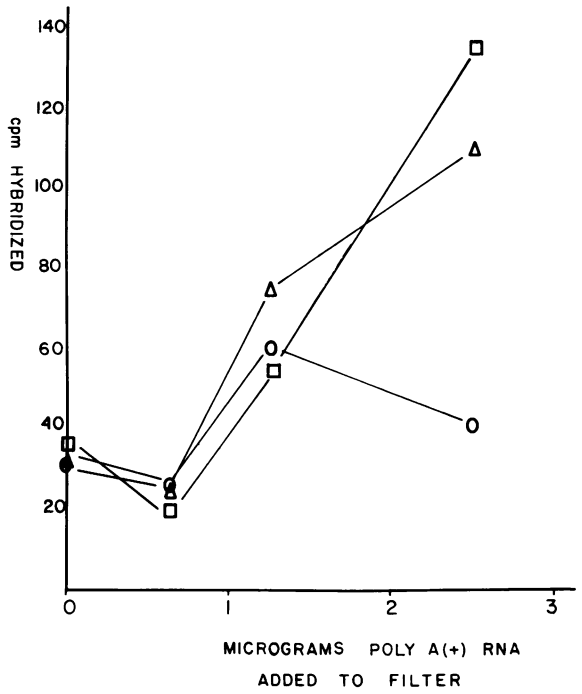

FIG. 2. Analysis of RNA from Raji cells electroporated with pSV2CAT (□), pFRCAT (△), or $pA_{10}CAT_2$ (○). At 72 h after electroporation, extracts from 10^7 cells were assayed for CAT activity, and total RNA was extracted from the remaining 10^8 cells. Poly(A)$^+$ RNA was selected, diluted, denatured, and applied to nitrocellulose. The filters were hybridized to ^{32}P-labeled pSV2CAT DNA to detect CAT RNA. The filters were washed and subjected to scintillation counting. The graph shows the counts per minute bound. Background hybridization to nitrocellulose alone (95 cpm) has been subtracted. The points for 0 RNA represent 2.5 μg of poly(A)$^+$ RNA from each cell population that had been treated with 0.6 N NaOH at 48°C for 2 h before its being applied to the filter.

tured salmon sperm DNA per ml. Hybridizations to ^{32}P-labeled DNA (2×10^8 to 4×10^8 cpm/μg) were carried out at 68°C for 18 h in the prehybridization mix. The filters were washed with 1× SSC–0.1% sodium dodecyl sulfate at 68°C and subjected to autoradiography and scintillation counting.

RESULTS

Family of 30-bp repeats from *oriP* enhances expression of CAT gene in cells that carry the EBV genome. We have found that the 30-bp family of repeats from *oriP* acts to enhance CAT expression approximately 25-fold from the SV40 early promoter in the EBV-genome-positive Burkitt lymphoma cell line Raji (36). To determine whether this activity of the 30-bp family of repeats is specific for EBV-positive cells or is a more general property of B-lymphoid cells, we tested the levels of CAT expressed from a series of CAT vectors introduced into both EBV-genome-positive and -genome-negative B-lymphoid cell lines. The structures of these plasmids that can express CAT are shown in Fig. 1A. The 30-bp family of repeats enhanced expression of the CAT gene only in cells that were EBV positive (Table 1). This enhanced CAT expression was observed when the 30-bp repeats were cloned in either orientation 200 bp upstream of the SV40 early promoter or approximately 2,000 bp away from the HSV-1 TK promoter. We conclude from these findings that the 30-bp family of repeats enhances the expression of the linked CAT gene. Since this activity could only be demonstrated in EBV-genome-positive cells, it is likely that an EBV-encoded gene product expressed in EBV-genome-positive cells acts in *trans* to permit this enhanced expression.

To determine whether the observed enhancement of gene expression by the 30-bp family of repeats as measured by levels of CAT activity reflected increased levels of CAT mRNA, we measured the amount of steady-state CAT mRNA in Raji cells into which we had introduced pSV2CAT, $pA_{10}CAT_2$, or pFRCAT (Fig. 1A). At 72 h after electroporation, 10^7 cells were assayed for CAT expression, and total RNA was extracted from the remaining cells. Poly(A)$^+$ RNA was selected by hybridization to polyurydilic acid paper. Twofold dilutions of the poly(A)$^+$ RNA were denatured, applied to nitrocellulose filters, and hybridized to ^{32}P-labeled pSV2CAT. CAT assays indicated that both pSV2CAT and pFRCAT yielded 2.0% acetylated chloramphenicol, while the vector $pA_{10}CAT_2$ yielded a background level of 0.1%. We observed a threefold increase in the level of CAT poly(A)$^+$ RNA in the cells expressing an increased level of CAT activity (Fig. 2). The levels of two EBV transcripts were measured by hybridizing poly(A)$^+$ RNA

TABLE 2. CAT expression from recombinant plasmids in EBNA-1-positive and EBNA-1-negative cell lines[a]

Cell line	EBNA-1 expression	Avg % chloramphenicol acetylated from extracts of cells carrying plasmids:						
		$pA_{10}CAT_2$	pSV_2CAT	pFRCAT	Ratio pFRCAT/$pA_{10}CAT_2$	pTKCAT	pFRTKCAT	Ratio pFRTKCAT/pTKCAT
A. CAT expression from SV40 early promoter								
Wilson/p205	+	0.1	7	2	20 (2)			
Wilson	−	0.1	11	0.1	1 (3)			
D98/p154	+	0.2	4	0.2	1 (1)			
D98	−	0.2	7	0.2	1 (1)			
143/EBNA	+	0.2	29	0.2	1 (3)			
143	−	0.2	30	0.2	1 (2)			
B. CAT expression from HSV-1 TK promoter								
Wilson/p274.4	+					0.1	4.8	48 (3)
Wilson	−					0.1	0.1	1 (1)
D98/p154	+					0.25	1.5	6 (3)
D98	−					0.2	0.2	1 (1)
143/EBNA	+					0.3	5.5	18 (2)
143	−					0.1	0.1	1 (1)

[a] The CAT recombinant plasmids listed were introduced into the cells listed and analyzed for CAT activity 72 h later as described in Table 1, footnote *a*. Plasmids were introduced into Wilson cells and their derivatives by electroporation and into all other cells by calcium phosphate coprecipitation.

TABLE 3. CAT expression mediated by different promoters and enhancers in EBNA-1-negative and -positive cells[a]

Cell line	EBNA-1	% Chloramphenicol actylated from extracts of cells carrying plasmid:				
		$pA_{10}CAT_2$	pTKCAT	pSV_2CAT	pCATwt760	pFRTKCAT
Wilson	−	0.2	0.2	3	11	0.2
Wilson p274.4	+	0.2	0.2	2.6	9	2
Ratio of activity in cells (EBNA-1 +/EBNA-1 −)		1	1	0.9	0.8	10

[a] Shown is the percent chloramphenicol acetylated by extracts of 10^7 Wilson (EBNA-1 negative) or Wilson/p274.4 (EBNA-1 positive) cells. The assays were performed as described in Table 1, footnote *a*, but the incubations were continued for 45 min at 37°C. Introduced plasmids were: $pA_{10}CAT_2$, SV40 early promoter lacking the enhancer; pTKCAT, HSV-1 TK promoter; pSV_2CAT, SV40 early promoter plus SV40 enhancer; pCATwt760, HCMV immediate-early promoter plus enhancer; pFRTKCAT, 30-bp family of repeats plus HSV-1 TK promoter.

labeled in vitro to DNA clones of EBV coding sequences (data not shown). While CAT RNA was observed to increase in Raji cells electroporated with either pSV2CAT or pFRCAT, levels of endogenous EBV mRNA transcripts were unchanged. These results indicate that the increase in CAT activity observed in EBV-genome-positive cells after the introduction of plasmids carrying the CAT gene and either the SV40 enhancer or the *oriP* family of repeats is likely to be due to an increase in transcription of the CAT gene. The insensitivity of this assay in detecting CAT poly(A)$^+$ RNA makes it impossible to determine whether or not the increase in this RNA synthesized from pSV2CAT and pFRCAT parallels exactly the observed increase in CAT enzyme activity.

***trans*-activation of the *oriP* transcriptional enhancer by the EBNA-1 gene product.** A number of experimental observations indicate that the EBNA-1 gene product of EBV interacts with the 30-bp family of repeats. The EBNA-1 gene product is required for the replication of plasmids containing *oriP* (29, 48). In addition, selectable plasmids that carry only the 30-bp family of repeats confer drug resistance transiently in EBNA-1-positive cells (36). More compellingly, Rawlins et al. (34) have shown by nuclease protection experiments that in vitro EBNA-1 binds to each copy of the 30-bp repeats.

We therefore asked whether EBNA-1 could act as a *trans*-activator of the 30-bp repeat enhancer element. To address this question, we generated cell lines which expressed EBNA-1 by introducing plasmids that carry the EBNA-1 gene plus a selectable drug resistance marker into EBV-genome-negative cells and selecting clones of cells for drug resistance. It is known that these EBV-genome-negative cell lines (Table 2) express EBNA-1 since the plasmids p205 and p154 carry *oriP* and are maintained as replicating plasmids. *oriP* plasmid maintenance requires the presence of EBNA-1. For the 143/EBNA and Wilson/p274 cells, the EBNA-1 gene is maintained by virtue of its being integrated into the host genome.

These EBNA-1-positive cell lines plus their EBNA-1-negative parents were tested for the ability to support transcriptional enhancement by the 30-bp repeats. The CAT constructions shown in Fig. 1A and listed in Table 2 were introduced into the cells, and after 72 h extracts were prepared and assayed for CAT activity (Table 2). In all the cell lines tested, pSV_2CAT yielded expression of the CAT gene, indicating that these cells permit the expression of the introduced DNA. Cells that did not express EBNA-1 did not support detectable transcriptional enhancement by the 30-bp repeats. Cells that express EBNA-1 were found to support transcriptional enhancement by the 30-bp repeats, but detectable expression of CAT activity depended on the promoter used. For example, in the EBV-negative, EBNA-1-positive, B-lymphoid line Wilson/p205, a 15- to 20-fold enhancement of CAT activity was observed when the 30-bp repeats were present on $pA_{10}CAT_2$ (Table 2A). $pA_{10}CAT_2$ contains the SV40 early promoter. However, we have previously shown that the 30-bp repeats do not detectably enhance transcription in 143/EBNA cells from the SV40

TABLE 4. Assay for stable enhancement: enumeration of hygromycin B-resistant clones per 10^6 cells after the introduction of different plasmids[a]

Cell line[b]	Plasmid tested[c]							Ratio[d] (p2.8/pHyg)
	pHyg	pHEBo	p2.8	p3.2	pSM3	p259	p260	
143 (150 μg)	4×10^2	6[e]	3×10^2	ND[f]	ND	ND	90	0.7
143/EBNA (150 μg)	1×10^2	4×10^3	10^3	ND	ND	ND	2×10^2	10
Raji (100 μg)	1	1×10^3	100	12	ND	12	12	100
Raji (200 μg)	1	ND	12	0.4	7×10^3	3	0.8	12
721 (200 μg)	1	3×10^2	1×10^2	3	7×10^2	9	1	100
721 (300 μg)	0.3	70	12	0.3	7×10^2	0.3	0.3	40

[a] Adherent cells (143 and 143/EBNA) were transfected and B-lymphoid cells (Raji and 721) were electroporated with plasmids that encode resistance to hygromycin B (Fig. 1B). The surviving cells were counted 2 to 3 days after introduction of plasmid DNAs, diluted, and treated with different concentrations of hygromycin B. Hygromycin B-resistant colonies were scored after 6 to 7 weeks.

[b] The concentration of hygromycin B used to select survivors is given in parentheses.

[c] For adherent cells the number of drug-resistant clones was determined by counting the number of colonies in dishes that had fewer than 50 colonies per dish and averaging those numbers for each plasmid tested. Adherent cells and B lymphoblasts were plated as described in Materials and Methods. The number of drug-resistant clones of B lymphoblasts was determined by counting the number of wells without live cells of a particular dilution. The Poisson distribution was then used to calculate the number of viable cells at that dilution. When more than one dilution had wells with and without live cells, then the average number of viable cells obtained from both dilutions is given.

[d] Ratio of the number of hygromycin B-resistant cells after the introduction of p2.8 divided by the number found after the introduction of pHyg.

[e] The number of hygromycin B-resistant 143 cells after introduction of pHEBo is usually similar to that found after introduction of pHyg (41, 48). This low number may reflect an inhibitor in this batch of pHEBo DNA used in this experiment with both 143 and 143/EBNA cells.

[f] ND, Not done.

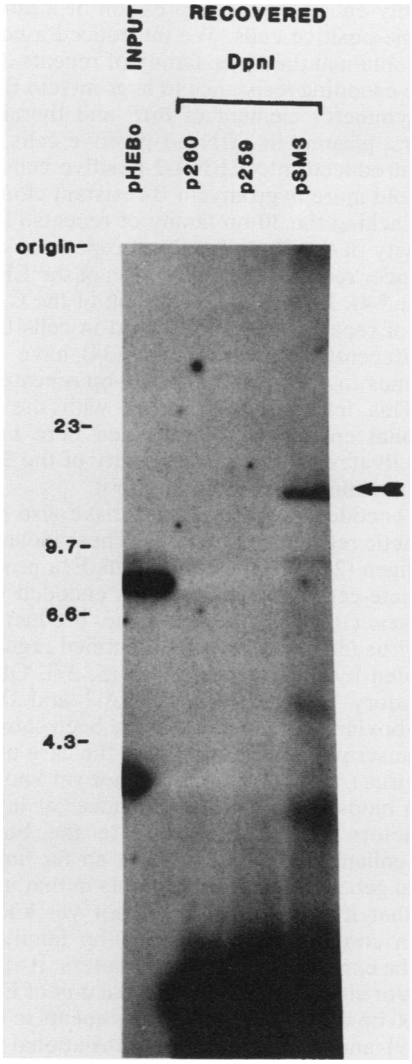

FIG. 3. Assay for replication of plasmids containing the SV40 enhancer. Raji cells (5×10^7) were electroporated with 10 μg of pSM3 (defective SV40 origin plus all of *oriP*), p259 (SV40 enhancer plus region of dyad symmetry from *oriP*), and p260 (SV40 enhancer alone). After 96 h, extracts from 10^7 cells were assayed as described in Materials and Methods. The arrow indicates the position of *Dpn*I-resistant and therefore replicated pSM3. The DNA in the lane labeled input was 30 pg of *Hin*dIII-digested pHEBo. Assuming that approximately 5% of the cells took up and expressed the DNA, 30 pg represents approximately 8 molecules per cell. The size markers (kilobase pairs) were taken from bacteriophage lambda DNA digested with *Hin*dIII. The autoradiogram was exposed for 4 days.

early promoter (36). This observation was confirmed and extended to D98 cells that express EBNA-1, D98/p154 (Table 2A). In contrast, when the CAT gene was placed under the transcriptional control of the HSV-1 TK promoter, the family of 30-bp repeats enhanced CAT expression approximately 5- to 20-fold in all cells tested that expressed EBNA-1 (Table 2B). Since the enhanced expression of genes linked to the 30-bp family of repeats is observed only in cells that express EBNA-1 and since EBNA-1 is known to bind to DNA sequences within each copy of the repeat, we conclude that EBNA-1 acts in *trans* to activate this enhancer element.

EBNA-1 *trans*-activates the 30-bp family of repeats and not other promoters or enhancers. We tested whether EBNA-1 acts as a specific activator of the 30-bp repeats or as a more general *trans*-activator of enhancer-promoter combinations. Five plasmids that expressed the CAT gene from different promoters were tested for their ability to express the CAT gene in cells either expressing or not expressing EBNA-1. The plasmids tested contained the CAT gene expressed from: the HSV-1 TK promoter (pTKCAT), the HSV-1 TK promoter plus the 30-bp family of repeats (pFRTKCAT), the SV40 early promoter plus the SV40 enhancer (pSV_2CAT), the SV40 early promoter ($pA_{10}CAT_2$), and the human cytomegalovirus immediate-early gene promoter plus enhancer (pCATwt760 [40]). These plasmids were introduced into Wilson (EBNA-1 negative) and Wilson/p274.4 (EBNA-1 positive) cells by electroporation. After 72 h, extracts were prepared and assayed for CAT activity. The results are presented in Table 3. In Wilson cells, introduction of both pSV_2CAT and pCATwt760 led to enhanced CAT expression (3 and 11% acetylation, respectively). Introduction of plasmids containing the 30-bp family of repeats led to no detectable CAT activity in these cells. In the EBNA-1-positive derivative of Wilson, Wilson/p274.4, both pSV_2CAT and pCATwt760 expressed levels of CAT activity similar to those observed in EBNA-1-negative Wilson cells (2.6 and 9% acetylation, respectively). CAT expression was enhanced from the plasmid carrying the 30-bp family of repeats, pFRTKCAT, in Wilson/p274.4 cells (2% acetylation). This effect was not observed for any of the other plasmids tested. These results indicate that EBNA-1 specifically *trans*-activates the 30-bp family of repeats which leads to enhanced expression of the CAT gene.

Family of 30-bp repeats stably enhances expression of linked drug resistance gene in EBNA-1-positive cells. We asked whether the 30-bp family of repeats could stably enhance expression of resistance to hygromycin B in addition to its ability to enhance gene expression transiently. The newly constructed plasmids used in these experiments are illustrated in Fig. 1B. They all express resistance to hygromycin B and in addition contain: the 30-bp family of repeats of *oriP* (p2.8 [36]); the dyad symmetry element of *oriP* (p3.2 [36]); *oriP* (pHEBo [41]); *oriP* plus the SV40 72-bp repeats (pSM3); the dyad symmetry element of *oriP* plus the SV40 72-bp repeats (p259); or the SV40 72-bp repeats alone (p260). These constructions were introduced into four different cell lines, and the cells were selected for resistance to hygromycin B. The number of cells resistant to hygromycin B per 10^6 viable cells is shown in Table 4. In 143 cells (EBNA-1 negative), the plasmid carrying the 30-bp family of repeats (p2.8) did not yield an increase in the number of resistant cells relative to the vector, pHyg. However, in cells that were positive for EBNA-1 expression (143/EBNA) or in cells that carried a complete EBV genome (Raji, 721), introduction of the plasmid p2.8 yielded cells resistant to hygromycin B 8 to 100 times more frequently than did introduction of the plasmid pHyg. Introduction of plasmids carrying all of *oriP* (pHEBo and pSM3) yielded a 10- to 100-fold-greater increase in the number of cells surviving selection than did introduction of p2.8. Plasmids that contained the dyad symmetry element of *oriP* (p3.2), the SV40 72-bp repeats (p260), or both (p259) yielded only 1/10th to 1/5th the number of hygromycin B-resistant cells as did p2.8. These observations indicate that the presence of the 30-bp family of repeats in *cis* enhances expression of the gene encoding hygromycin B resistance in cells that express EBNA-1. Furthermore, the enhancement by the 30-bp family of repeats is more efficient than that observed with the SV40 72-bp repeats.

Increasing the stringency of the selection by increasing the dose of hygromycin B usually led to a decrease in the number of resistant cells recovered (Table 4). p2.8 still yielded more resistant cells than did the vector under these more stringent selection conditions. This observation is again consistent with the 30-bp family of repeats stably enhancing expression of a linked gene. With p2.8 increasing the stringency of selection still further led to colonies being resistant only transiently (36). The transient survival results from high levels of hygromycin B resistance being expressed transiently. This transient resistance results in turn presumably from more molecules of p2.8 being introduced and expressed in cells than are eventually maintained and expressed.

SV40 transcriptional enhancer cannot substitute for the 30-bp repeats in *oriP*-mediated plasmid replication. We found that the 30-bp repeats of *oriP*, which is required for plasmid replication, also can function as a transcriptional enhancer. A transcriptional enhancer and a region containing dyad symmetry are both required for the efficient replication of papovaviruses, and at least with polyomavirus, this requirement for a transcriptional enhancer can be met by heterologous enhancers (9, 18a). We therefore tested whether an SV40 transcriptional enhancer could substitute for the family of 30-bp repeats in *oriP* replication. To carry out this test, we constructed three plasmids with the SV40 enhancer, *oriP*, or only the region of dyad symmetry from *oriP* (illustrated in Fig. 1B). pSM3 (SV40 origin plus all of *oriP*), p259 (SV40 enhancer plus dyad symmetry from *oriP*), and p260 (SV40 enhancer) were introduced into Raji cells and after 96 h were assayed for replication as described above. Visualization of the autoradiogram (Fig. 3) indicated that the only plasmid that had undergone replication was pSM3 which contained all of *oriP*. pSM3 was present at a level of approximately two replicated plasmid molecules per cell, and the limit of detection was less than 0.4 replicated molecules per cell. This result indicates that unlike polyomavirus DNA replication, efficient replication of *oriP* plasmids cannot be rescued by substituting a heterologous transcriptional enhancer for the EBV 30-bp family of repeats. The results of this assay for replication are corroborated by the findings in Table 4. DNAs encoding resistance to hygromycin B that contain *oriP* (pHEBo and pSM3) yield approximately 1,000-fold more resistant colonies after being introduced into EBNA-1-positive cells than do the constructions p259 and p260. The failure of p259 and p260 to yield resistant colonies efficiently results presumably from both their lack of the 30-bp family of repeats that serves as a transcriptional enhancer and their inability to replicate as plasmids.

DISCUSSION

The 30-bp family of repeats of *oriP* can function as a transcriptional enhancer. When positioned upstream of the CAT gene expressed from the SV40 early promoter or approximately 2,000 bp downstream from the CAT gene expressed from the HSV-1 TK promoter, the 30-bp family of repeats enhances CAT expression in EBV-genome-positive or EBNA-1-positive cells. This enhanced CAT expression was found to reflect an increased level of CAT mRNA and was not due to plasmid amplification. The plasmids do not amplify because those that contain only the 30-bp repeats of *oriP* have been shown not to replicate in a short-term assay (36), and in an analogous experiment the plasmids $pA_{10}CAT$ and pFRCAT were found not to replicate (data not shown). We found by using a long-term assay that the 30-bp family of repeats stably enhanced the expression of a linked gene in EBV-genome-positive cells. We introduced a construction, p2.8, that contained the 30-bp family of repeats downstream from a gene encoding resistance to hygromycin B. p2.8 lacks the dyad symmetry element of *oriP* and therefore cannot replicate as a plasmid in EBNA-1-positive cells (36). When p2.8 was introduced into EBNA-1-positive cells, it yielded 10- to 100-fold more hygromycin B-resistant clones than did the vector lacking the 30-bp family of repeats (Table 4).

The activity of the 30-bp family of repeats as a transcriptional enhancer requires the expression of the EBNA-1 gene (Tables 2 and 4). Enhanced expression of the CAT gene by the family of repeats is only observed in cells that express EBNA-1. Recently, Rawlins et al. (34) have shown that EBNA-1 binds to each copy of the 30-bp repeated sequence in vitro. This information together with the studies on transcriptional enhancement presented here indicate that EBNA-1 activates the enhancer activity of the 30-bp family of repeats, presumably, by binding to it.

Proteins encoded by other viruses have also been shown to have genetic regulatory functions. These include the SV40 large T antigen (23, 30), the adenovirus E1a protein (3, 21), the immediate-early class of proteins encoded by the lytic herpesviruses (10, 32), the E2 gene product of bovine papillomavirus (38), and recently identified regulatory functions encoded by some retroviruses (5, 37). Of this list of viral regulatory proteins only EBNA-1 and the E2 gene product of bovine papillomavirus have been shown to *trans*-activate transcriptional enhancers. In the case of the bovine papillomavirus *trans*-activation, it is not yet known whether the protein binds directly to the enhancer or interacts with cellular factors which then activate the bovine papillomavirus enhancer (38). EBNA-1 is so far unique among known viral genetic regulatory proteins in that it binds to an enhancer that it activates. We do not yet know whether EBNA-1 in conjunction with the 30-bp family of repeats enhances the expression of EBV promoters. It appears likely that the major clockwise transcriptional unit of EBV initiates within 4,000 bp of the 30-bp family of repeats in transformed cells (4, 39) and that one of its translational products is EBNA-1 itself (39). If the expression of this transcriptional unit were to be enhanced by the 30-bp family of repeats, then the EBNA-1 protein would activate its own synthesis.

EBNA-1 may also activate the expression of cellular genes. If there are clustered DNA sequences in the human genome to which EBNA-1 binds as it does to the 30-bp family of repeats, then this binding may enhance the expression of nearby genes. One candidate gene whose enhanced expression may be activated by EBNA-1 is the proto-oncogene homologous to the Gardner-Rasheed feline sarcoma virus. This proto-oncogene is induced both in EBV-transformed cells and in EBV-negative Burkitt lymphoma cell lines that have been infected in vitro with EBV (6).

Both the EBNA-1 protein and the 30-bp family of repeats are required for the replication of plasmids that contain *oriP*. Transcriptional enhancers have been found to be required for the replication of SV40 and polyomavirus. However, the DNA sequences involved in transcription and DNA replication for these two papovaviruses are nested and therefore difficult to dissect functionally. Whether or not the capacity of the 30-bp family of repeats to act as a transcriptional enhancer is required for DNA replication mediated by *oriP* is unknown. However, another enhancer, the SV40 72-bp enhancer, cannot substitute for the EBV 30-bp family of repeats to permit efficient replication of *oriP* (Table 4; Fig. 3). It is likely that some step in DNA replication and RNA

transcription involves the action of common *cis*-acting DNA sequences since the 30-bp family of repeats can affect both DNA replication and transcriptional enhancement. The viral protein EBNA-1 is required for both processes too, and presumably meets its requirement by binding to the 30-bp family of repeats and, perhaps, to various cellular regulatory factors.

ACKNOWLEDGMENTS

We thank C. Chen-Kiang for the ManCa cell line. We thank our colleagues Vijay Baichwal, Gerry Hertz, Joyce Knutson, Stan Metzenberg, Howard Temin, and John Yates for their experimental suggestions and criticisms of the manuscript.

This work was supported by Public Health Service grants CA-22443, CA-07175, and T32CA09135 from the National Institutes of Health.

LITERATURE CITED

1. **Arrand, J. R., and L. Rymo.** 1982. Characteristics of the major Epstein-Barr virus-specific RNA in Burkitt lymphoma-derived cells. J. Virol. **41**:376–389.
2. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Dieninger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Sequin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
3. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
4. **Bodescot, M., B. Chambraud, P. Farrell, and M. Perricaudet.** 1984. Spliced RNA from the 1R1-U2 region of Epstein-Barr virus: presence of an open reading frame for a repetitive polypeptide. EMBO J. **3**:1913–1917.
5. **Broome, S., and W. Gilbert.** 1985. Rous sarcoma virus encodes a transcriptional activator. Cell **40**:537–546.
6. **Cheah, M. S. C., T. J. Ley, S. R. Tronick, and K. C. Robbins.** 1986. *fgr* proto oncogene mRNA induced in B-lymphocytes by Epstein-Barr virus infection. Nature (London) **319**:238–240.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
8. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
9. **de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen.** 1984. Polyoma virus DNA replication requires an enhancer. Nature (London) **312**:242–246.
10. **Dixon, R. A. F., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP 175. J. Virol. **36**:189–203.
11. **Fennewald, S., V. van Santen, and E. Kieff.** 1984. Nucleotide sequence of an mRNA transcribed in latent growth transforming virus infection indicates that it may encode a membrane protein. J. Virol. **51**:411–419.
12. **Fromm, M., and P. Berg.** 1982. Deletion mapping of DNA regions required for SV40 early promoter function in vivo. J. Mol. Appl. Genet. **1**:457–481.
13. **Glaser, R., and M. Nonoyama.** 1973. Host cell replication and induction of Epstein-Barr virus. J. Virol. **14**:174–176.
14. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
15. **Graham, F. L., S. Bacchetti, R. McKinnon, B. Cordell, and H. Goodman.** 1979. Transformation of mammalian cells with DNA using the calcium technique. *In* R. Basergan (ed.), Introduction of macromolecules into viable mammalian cells. Wistar Symposium Series, vol. 1. Alan R. Liss, Inc., New York.
16. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **5**:456–467.
17. **Hennessey, K., M. Heller, V. van Santen, and E. Kieff.** 1983. Simple repeat array in Epstein-Barr virus DNA encodes part of the Epstein-Barr nuclear antigen. Science **220**:1396–1399.
18. **Hennessey, K., and E. Kieff.** 1985. A second nuclear protein is encoded by Epstein-Barr virus in latent infection. Science **227**:1238–1240.

18a. **Hertz, G., and J. Mertz.** 1986. Bidirectional promoter elements of simian virus 40 are required for efficient replication of viral DNA. Mol. Cell. Biol. **6**:3513–3522.

19. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
20. **Hudson, G. S., A. T. Bankier, S. C. Satchwell, and B. Baverell.** 1985. The short unique region of the B95-8 Epstein-Barr virus genome. Virology **147**:1–98.
21. **Jones, N., and T. Shenk.** 1979. An adenovirus 5 early gene product function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA **76**:3665–3669.
22. **Kavathas, P., F. H. Bach, and R. DeMars.** 1980. Gamma ray-induced loss of expression of HLA and glyoxalase alleles in lymphoblastoid cells. Proc. Natl. Acad. Sci. USA **77**:4251–4255.
23. **Keller, J. M., and S. C. Alwine.** 1984. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication. Cell **36**:381–389.
24. **Klein, G., B. Giovanella, A. Westman, J. S. Stehlin, and D. Mumford.** 1975. An EBV-genome negative cell line established from an American Burkitt lymphoma; receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by in vitro infection. Intervirology **5**:319–334.
25. **Klein, G., T. Lindahl, M. Jondal, W. Leibold, J. Meneles, K. Nilsson, and C. Sundström.** 1974. Continuous lymphoid cell lines with characteristics of B-cells (bone marrow derived), lacking the Epstein-Barr virus genome and derived from three human lymphomas. Proc. Natl. Acad. Sci. USA **71**:3283–3286.
26. **Laimins, A. L., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
27. **Lindahl, T., A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich, and U. Jehn.** 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J. Mol. Biol. **102**:511–530.
28. **Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
29. **Lupton, S., and A. J. Levine.** 1985. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. Mol. Cell. Biol. **5**:2533–2542.
30. **Myers, R. M., D. C. Rio, A. K. Robbins, and R. Tjian.** 1981. SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. Cell **25**:373–384.
31. **Miller, G.** 1985. Epstein-Barr virus, p. 563–590. *In* B. Fields (ed.), Virology. Raven Press, New York.
32. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsk*. J. Virol. **29**:275–284.
33. **Pulvertaft, R. J. V.** 1965. A study of malignant tumors in Nigeria by short-term tissue culture. J. Clin. Pathol. **18**:261–271.
34. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42**:859–868.
35. **Reedman, B. M., and G. Klein.** 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer **11**:499–520.
36. **Reisman, D., J. Yates, and B. Sugden.** 1985. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting components. Mol. Cell. Biol. **5**: 1822–1832.
37. **Sodroski, J. G., W. C. Goh, C. A. Rosen, S. Z. Salahuddin, A.**

Aldouini, G. Franchini, F. Wong-Staal, R. C. Gallo, K. Sugamura, Y. Hinuma, and W. A. Haseltine. 1985. *Trans*-activation of the human T-cell leukemia virus long terminal repeat correlates with expression of the *x-los* protein. J. Virol. **55**:831–835.

38. **Spalholz, B. A., Y. C. Yang, and P. M. Howley.** 1985. *Trans*activation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
39. **Speck, S. H., and J. L. Strominger.** 1985. Analysis of the transcript encoding the latent Epstein-Barr virus nuclear antigen. I. A potentially polycistronic message generated by long-range splicing of several exons. Proc. Natl. Acad. Sci. USA **82**:8205–8309.
40. **Stinski, M. F., and T. J. Roehr.** 1985. Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulating sequence and by virus-specific *trans*-acting components. J. Virol. **55**:431–441.
41. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid can be efficiently selected in B-lymphoblasts transformed by EBV. Mol. Cell. Biol. **5**:410–413.
42. **Sugden, B., M. Phelps, and J. Domoradski.** 1979. Epstein-Barr virus DNA is amplified in transformed lymphocytes. J. Virol. **31**:590–595.
43. **Summers, W. P., E. A. Grogan, D. Shedd, M. Robert, C. Liu, and G. Miller.** 1982. Stable expression in mouse cells of nuclear neoantigen after transfer of a 3.4-megadalton cloned fragment of Epstein-Barr virus DNA. Proc. Natl. Acad. Sci. USA **79**: 5688–5692.
44. **Szybalski, W., E. H. Szybalska, and G. Ragni.** 1962. Genetic studies with human cell lines. Natl. Cancer Inst. Monogr. **7**:75–89.
45. **van Santen, V., A. Cheung, and E. Kieff.** 1981. Epstein-Barr virus RNA. VII. Size and direction of transcription of virus-specific cytoplasmic RNAs in a transformed cell line. Proc. Natl. Acad. Sci. USA **78**:1930–1935.
46. **White, B., and F. C. Bancroft.** 1982. Cytoplasmic dot hybridization. J. Biol. Chem. **257**:8569–8572.
47. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81**:3806–3810.
48. **Yates, J. L., N. Warren, and B. Sugden.** 1985. Plasmids derived from Epstein-Barr virus replicate stably in a variety of mammalian cells. Nature (London) **313**:812–815.