JOURNAL OF VIROLOGY, June 1990, p. 2876–2883
0022-538X/90/062876-08$02.00/0

Vol. 64, No. 6

Plasmid Origin of Replication of Herpesvirus Papio: DNA Sequence and Enhancer Function

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Herpesvirus papio (HVP) is a lymphotropic virus of baboons which is related to Epstein-Barr virus (EBV) and produces latent infection. The nucleotide sequence of the 5,775-base-pair (bp) *Eco*RI K fragment of HVP, which has previously been shown to confer the ability to replicate autonomously, has been determined. Within this DNA fragment is a region which bears structural and sequence similarity to the *ori-P* region of EBV. The HVP *ori-P* region has a 10- by 26-bp tandem array which is related to the 20- by 30-bp tandem array from the EBV *ori-P* region. In HVP there is an intervening region of 764 bp followed by five partial copies of the 26-bp monomer. Both the EBV and HVP 3′ regions have the potential to form dyad structures which, however, differ in arrangement. We also demonstrate that a transcriptional enhancer which requires transactivation by a virus-encoded factor is present in the HVP *ori-P*.

The episomal form of the Epstein-Barr virus (EBV) genome is thought to be the molecular basis for latent EBV infection (1, 25, 26, 28), but the mechanism of the persistence of the episome in cells was until recently largely unknown. EBV episomes are unit-length supercoiled intracellular EBV genomes found in non-virus-producing cells at copy numbers that are both limited and fixed in successive cell generations (19, 24, 28). The episomes are intranuclear and in a nucleosomal arrangement (37) and are thought to be replicated by host rather than viral polymerase (5, 28). Transcripts found spanning the fused genomic termini in infected cells indicate that at least one EBV gene in the episomal form is transcribed (17).

Replication of EBV episomes requires two *cis*-acting elements in the *Bam*HI-C region of the genome, which constitute the plasmid origin of replication (*ori-P*) (35, 38, 40, 41). The *cis*-acting elements consist of a series of repeat units located at the 5′ end of *ori-P* separated by intervening sequence from an area of dyad symmetry located at the 3′ end (20), where replication is initiated (8). Transactivation by the EBNA-1 protein is required for the episomal maintenance function of *ori-P*, as well as for a transcriptional enhancer activity that has been identified within the 5′ element of *ori-P* (34).

We have analyzed herpesvirus papio (HVP), a lymphotropic virus of baboons that is able to immortalize both human and baboon B lymphocytes in vitro (31), to generalize these mechanisms of episomal maintenance to another virus. HVP is similar structurally and biologically to EBV, but on the genomic level the viruses share only 40% homology (7, 10, 18). A region in HVP with sequence and functional homology to the EBV *ori-P* has been identified which requires a *trans*-acting function analogous to that found in EBV (29, 30). This transactivation can be supplied by either EBNA-1 or HVP protein, presumably HVP nuclear antigen (27). Conversely, constructs containing the EBV *ori-P* can be maintained in HVP-infected cells, although HVP nuclear antigen and EBNA-1 are not identical proteins (6). We therefore sequenced the HVP *ori-P* to identify and compare the essential conserved features of the two origins of replication. We also demonstrate that a transcriptional enhancer, requiring transactivation by a virus-encoded factor, exists within the HVP *ori-P*.

MATERIALS AND METHODS

Sequencing of the 5,775-base-pair (bp) HVP *Eco*RI K fragment. The HVP *Eco*RI K fragment was subcloned and sequenced as described by Bankier et al. (3). The sequence was compiled and analyzed with programs described by Staden (38).

Cell lines. B95-8, an EBV-productive marmoset B-cell line (21); Raji, a human Burkitt's lymphoma-derived line latently infected with EBV; Loukes, an EBV genome-negative human B-cell line; and 594-S (32), a baboon B-cell line productively infected with HVP, were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in a 5% CO_2 environment.

DNA transfections. Plasmid DNA was amplified in *Escherichia coli* and purified over two sequential cesium chloride gradients. For each sample, 10 μg of plasmid DNA was electroporated (23) into 10^7 cells at 1,500 V. Cells were then suspended in 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 48 h at 37°C in 5% CO_2.

Chloramphenicol acetyltransferase (CAT) assays. Extracts were prepared by washing cell pellets twice in phosphate-buffered saline solution, suspending them in 200 μl of 0.25 M Tris hydrochloride (pH 7.5), and freeze-thawing four times. Protein determinations were made by using the Bio-Rad Laboratories protein assay. All samples in each assay contained equal amounts of protein from extracts of 10^7 cells. Reactions were carried out as described previously (9) by incubating each sample with acetyl coenzyme A and [^{14}C]chloramphenicol at 37°C for 60 min. Acetylated reaction products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by scintillation counting.

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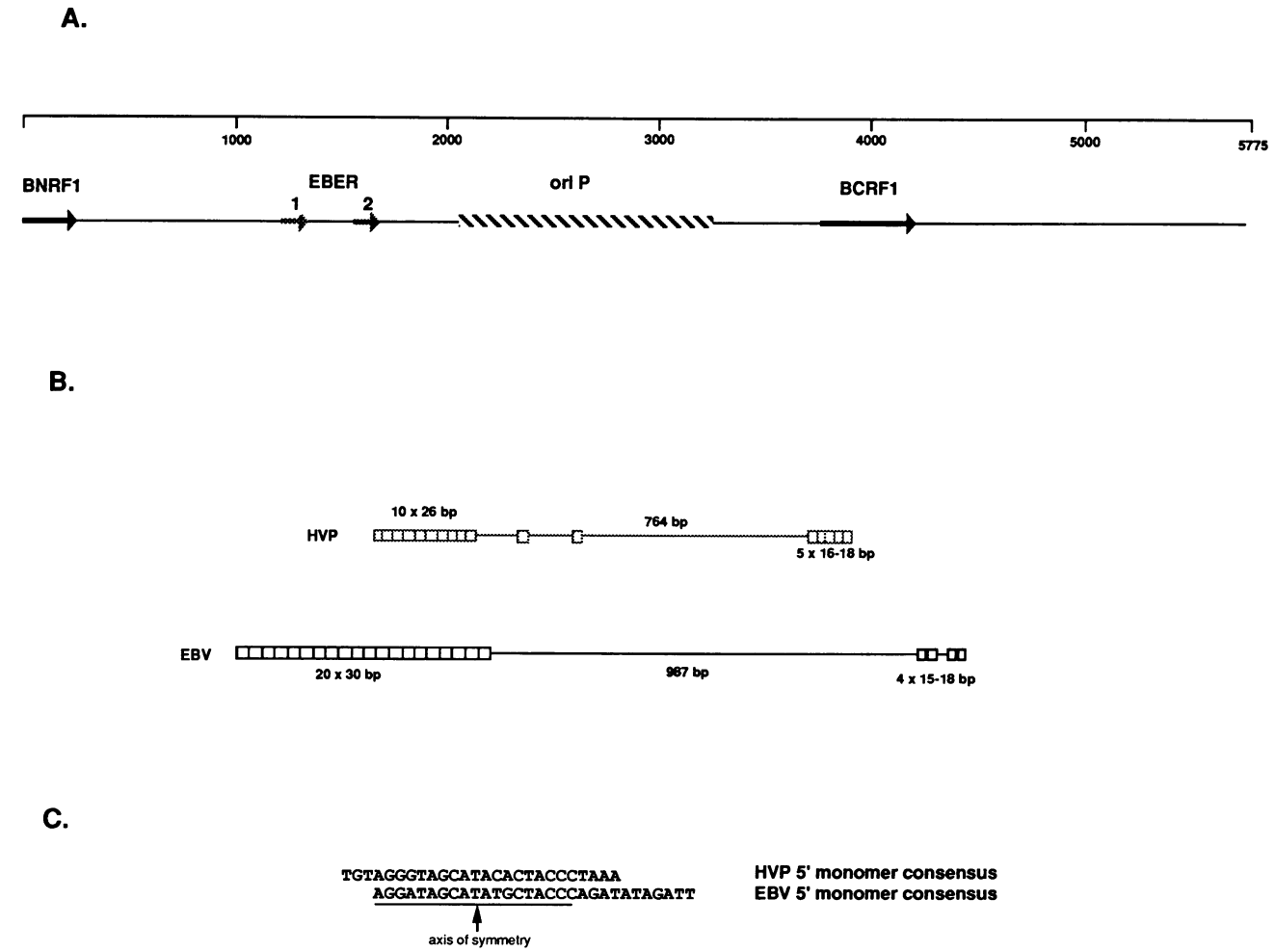

FIG. 1. HVP and EBV *ori-P* regions. (A) Map of sequence features in the HVP *Eco*RI K fragment. The following features are in common with EBV. Open reading frames and direction of transcription are denoted by black arrows. Bases 1 to 326 correspond to the 3′ end of the EBV BNRF1 reading frame. Bases 3770 to 4285 correspond to the EBV BCRF1 reading frame. EBER 1 and 2 are denoted by gray arrows and are found at bases 1235 to 1424 and 1549 to 1717, respectively. The *ori-P* region encompasses positions 2084 to 3220. (B) Organization of *ori-P* regions of EBV and HVP. The *ori-P* of each virus has the general structure of an array of monomer units, followed by an intervening sequence of variable length, followed by four or five partial copies of the monomer unit which has the potential to form a large dyad. HVP has an additional two partial copies of the monomer unit at the 5′ end of the intervening sequence. (C) Comparison of 5′ array monomer consensus sequences of EBV and HVP. Sequence common to EBV and HVP is underlined (match in 16 of 19 bp). Arrow indicates dyad of symmetry in each monomer unit. HVP consensus sequence was generated by considering only the sequences in the 5′ arrays. The EBV consensus sequence is taken from Fig. 6 of Lupton and Levine (20).

Plasmid constructions. $pA_{10}CAT$-680-1 and $pA_{10}CAT$-680-2 were generated by cloning a 680-bp DNA fragment from the HVP *ori-P* region, containing five of the repeat units as well as two imperfect repeat units from the intervening sequence, in both orientations, into the *Bam*HI site of $pA_{10}CAT$ (see Fig. 4). The $pA_{10}CAT$ vector contains the CAT gene under the control of the simian virus 40 (SV40) early promoter but lacks the SV40 enhancer sequences (16). pCAT3M680-1 and pCAT3M680-2 were generated by cloning the same 680-bp fragment, in both orientations, into the *Bgl*II site of the promoterless CAT vector pCAT3M (see Fig. 4) (15). A second construct containing the entire motif of 10 repeat units was also cloned into $pA_{10}CAT$.

RESULTS

Nucleotide sequence of the HVP *Eco*RI K fragment. The nucleotide sequence of the HVP *Eco*RI K fragment was determined. It is 5,775 bp in length and is homologous to sequences within the EBV *Bam*HI C fragment (nucleotides 5367 to 11635 of the EBV sequence). Within this region of the EBV genome there are five notable features: the 3′ portion of the BNRF1 reading frame; the entire BCRF1 reading frame; two polymerase III RNAs, EBER 1 and 2; and the *ori-P* region (Fig. 1A and B). All of these features are also found in the HVP *Eco*RI K fragment. The HVP EBER 1 and 2 are 78 and 66% homologous, respectively, to the EBV EBER sequence reported by Howe and Shu (11). Although the HVP *Eco*RI K fragment and its corresponding EBV sequence are homologous for most of their lengths, especially in these functional domains, there are regions in which there is no obvious sequence homology. Table 1 contains a summary of the degree of homology between the EBV and HVP sequences.

Sequence of the HVP *ori-P* region and comparison with the

TABLE 1. Sequence homology between the HVP *Eco*RI K fragment and the homologous region in EBV as a function of nucleotide position

HVP positions[a]	EBV positions[b]	% Homology[c]	EBV feature[d]
1–326	5367–5692	73	BNRF1
327–754	5693–6153	70	3′ to BNRF1
755–1050	6154–6406	NSH	
1051–1234	6407–6628	56	5′ to EBER 1
1235–1424	6629–6795	80	EBER 1
1425–1548	6796–6955	60	Intervening
1549–1717	6956–7128	66	EBER 2
1718–1810	7129–7206	54	3′ to EBER 2
1811–1990	7207–7302	NSH	
1991–2083	7303–7449	58	5′ to array
2084–2347	7450–8032	84	10- by 26-bp array
2348–2621	8033–8554	NSH	Intervening
2622–3090	8555–9032	50	Intervening
3134–3224	9033–9133	70	3′ dyad motif
3225–3611	9134–9515	NSH	
3612–3769	9516–9674	64	5′ to BCRF1
3770–4285	9675–10187	82	BCRF1
4286–4595	10188–10491	73	3′ to BCRF1
4596–4895	10492–10765	NSH	
4896–577	10766–11635	69	

[a] Refers to the nucleotide coordinate within the HVP *Eco*RI K fragment.
[b] Refers to the nucleotide ccordinate within the published B95-8 EBV sequence (2).
[c] Homology values within the 10- by 26-bp array refer to a match in 16 of 19 bp between the consensus EBV and consensus HVP monomer sequences. NSH, No significant homology found.
[d] Features are those previously described in the EBV B95-8 sequence.

EBV *ori-P*. Although the general format of the HVP *ori-P* is similar to that of the EBV *ori-P*, there are considerable differences between the two *ori-P*s on the nucleotide level. To facilitate the description of the HVP *ori-P* region, a brief review of the structural features of the EBV *ori-P* is in order. The 5′ end of the EBV *ori-P* region has 20 copies of a 30-bp unit in a tandem array. Approximately 987 bp downstream of this 20- by 30-bp tandem array are four partial copies of the 30-bp monomer. Dyad structures can be proposed in this downstream region, with monomers and surrounding sequence contributing to the structure (14, 20, 33).

The HVP *ori-P* is similar in its general structure, with the following specific differences (Fig. 1B). The 5′ end of the HVP *ori-P* region has 10 imperfect and/or partial copies of a 26-bp unit in tandem array. This 26-bp monomer is related to the EBV 30-bp monomer (Fig. 1C; see below). Approximately 764 bp downstream of the 10- by 26-bp array are five partial copies of the 26-bp unit. The intervening 764-bp sequence harbors two imperfect copies of the 26-bp monomer, which are not found in the EBV *ori-P*. As in EBV, the 3′ sequence of the HVP *ori-P* has the potential to form a large dyad (80 bp), but unlike in EBV, only monomer sequence contributes to the proposed dyad structure (Fig. 2).

Comparison of the EBV and HVP *ori-P* repeat units. The 30-bp EBV repeat monomer and the HVP 26-bp repeat monomer are homologous for 19 bp. A comparison of a consensus sequence of the repeat unit of each virus reveals a match in 16 of 19 bp (Fig. 1C). If the first base of these consensus sequences is discarded, an imperfect 9- by 9-bp dyad of symmetry can be formed, as previously noted for EBV. When only the HVP repeat unit is considered, this dyad can be extended to an imperfect 13- by 13-bp dyad, which comprises the entire 26-bp unit. In EBV the dyad cannot be extended. Thus in the HVP *ori-P*, all 26 bp of each monomeric unit can form an imperfect dyad, whereas in EBV only 18 bp of each 30-bp monomeric unit can form a dyad.

Examination of the pattern of polymorphisms within the individual EBV repeat units reveals an imperfect duplication of a putative ancestral 10- by 30-bp array leading to the present-day 20- by 30-bp array. In this respect, the 10- by 26-bp HVP array would be more similar to the ancestral EBV array in size.

Toward defining a recognition site for the HVP *trans*-acting protein. The HVP *ori-P*, like the EBV *ori-P*, requires activation by a *trans*-acting protein. Since transactivation appears to be brought about by actual binding of EBNA-1 to the repeat units (22, 33), the variations tolerated within the repeat monomers may be useful in further defining the sequence that the HVP transactivator recognizes. When only the 5′ tandem array of each virus is considered, 19 bp in the monomer unit is shared by both viruses (Fig. 3). Additionally, the portion of the monomer unit that is common between the 5′ and 3′ arrays within both viruses is the last 18 bp of the above-mentioned 19 bp. This 18-bp sequence is palindromic and contains the 12-bp core EBNA-1-binding sequence (12). A consensus sequence, GGRTAGYMTRYR CTRYCC, in which R is a purine, Y is a pyrimidine, and M

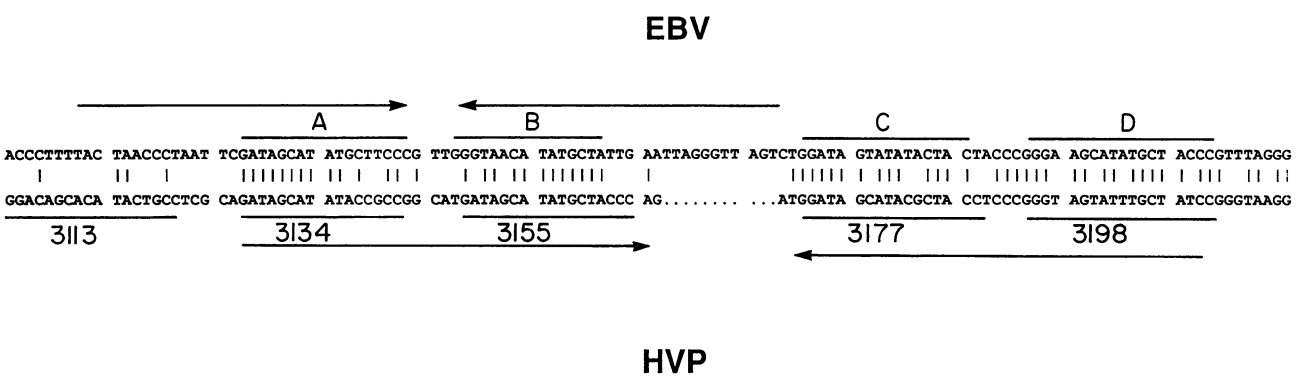

FIG. 2. Sequence comparison of the 3′ dyad regions of HVP and EBV. The upper nucleotide sequence is EBV; the lower sequence is HVP. Labeled overlined and underlined regions (A to D) indicate copies of the monomer unit. The regions of dyad symmetry are indicated by arrows. Although other EBV dyad structures are possible, that proposed by Lupton and Levine (20) is shown here. The indicated HVP dyad is the largest among several that can be formed.

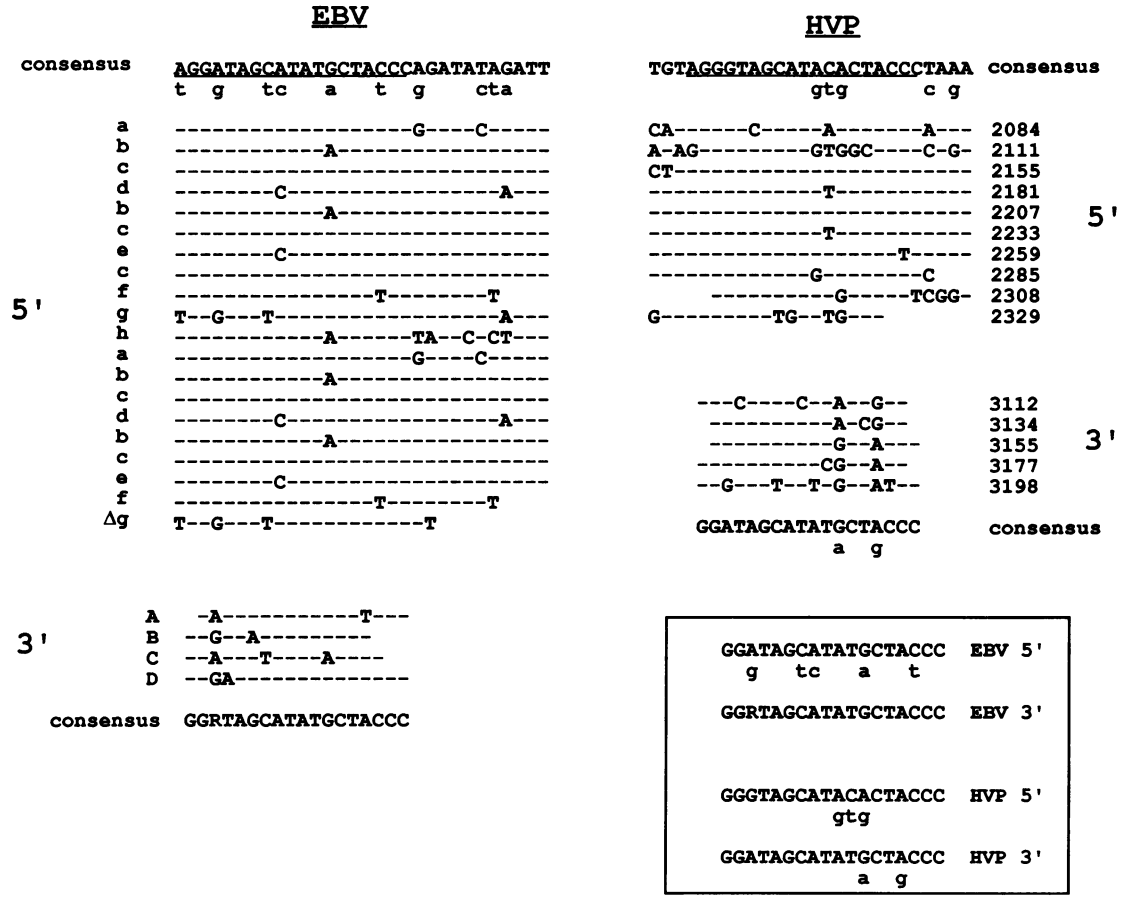

FIG. 3. Comparison of the monomer sequences in the HVP and EBV *ori-P* regions. The consensus sequence is generated by noting the most common nucleotide at that position. A consensus was generated for the 5′ and 3′ arrays for each virus. Nonconsensus nucleotides appearing in more than one monomer are denoted by lowercase letters beneath the consensus nucleotides. Aligned below the consensus sequence are the individual complete and partial monomer sequences, with differences indicated. Names appear next to each monomer. HVP monomers were identified by searching the HVP *Eco*RI K fragment for a match in 9 of 12 bp by the sequence TAGCATATGCTA. All but the monomer at position 3113 were identified by this method. The 3113 monomer was visually identified. EBV monomers were taken from Fig. 6 of Lupton and Levine (20). (Inset) Alignment of common nucleotides in the consensus sequences from the 5′ and 3′ arrays is shown. Minor variants are denoted by lowercase letters beneath the respective consensus sequence. R indicates a purine (A or G).

is A or C, can be derived from the EBV and HVP 18-bp palindrome.

Demonstration of a transcriptional enhancer element within the HVP *ori-P*. The EBV *ori-P* contains a transcriptional enhancer (34, 41). To determine whether enhancer activity could also be localized to the 5′ repeat element of the HVP *ori-P*, constructs were made containing HVP repeated units linked to the CAT reporter gene. A 680-bp fragment containing five of the repeat units and the two imperfect units from the intervening sequence was cloned in both orientations downstream of the CAT gene in the pA_{10}CAT vector (Fig. 4). This 680-bp fragment was able to enhance CAT expression from the SV40 early promoter by approximately 12-fold in 594-S cells expressing HVP gene products and by 9-fold in B95-8 cells expressing EBV gene products but was unable to enhance expression in EBV- or HVP-negative cells (Fig. 5 and Table 2).

To demonstrate that the enhancement observed was not due to promoter activity intrinsic to the 680-bp fragment, it was cloned in both orientations into the promoterless CAT vector pCAT3M (Fig. 4). No enhancement was observed in any of the cell lines tested. Representative assays are shown in Fig. 5.

Additional pA_{10}CAT constructs consisting of a 1,750-bp fragment (HVP *Eco*RI-K positions 1688 to 2438 in both orientations), which included all 10 repeat units in the motif but omitted the 2 repeats in the intervening sequence, were also tested. When they were assayed simultaneously in either EBV-infected or HVP-infected cells, the CAT activity

TABLE 2. Quantitation of enhancement of CAT expression in different cell lines

Cell line	Virus[a]		Fold enhancement[b]	
	EBV	HVP	680-1	680-2
Loukes	−	−	1.7 (8)	0.7 (7)
B95-8	+	−	9.3 (6)	8.9 (5)
594-S	−	+	13.9 (4)	11.8 (5)

[a] The presence (+) or absence (−) of viral genomes in each cell line is indicated.

[b] Ratio of the percent acetylation per microgram of extract in samples containing the 680-bp insert to the percent acetylation in samples containing the pA_{10} CAT vector alone, expressed as the average of the number of determinations indicated in parentheses. 680-1 and 680-2 designate the two orientations of the insert.

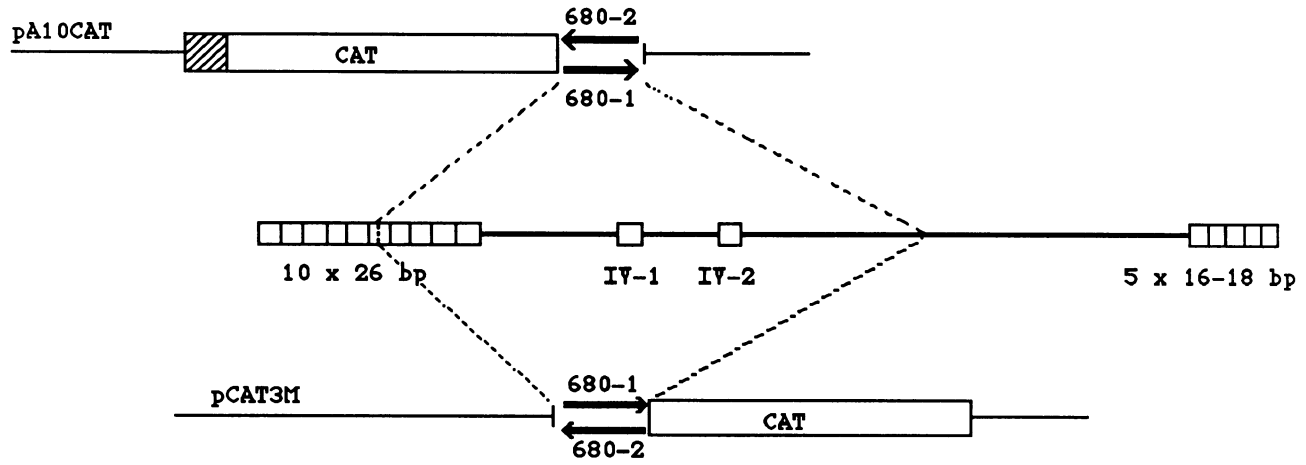

FIG. 4. Plasmid constructions containing a portion of the HVP repeat motif. pA_{10}CAT-680-1 and pA_{10}CAT-680-2 indicate the two orientations in which the 680-bp fragment was cloned into the *Bam*HI site directly downstream of the CAT gene in the vector pA_{10}CAT. The position of the SV40 early promoter is indicated (▨). pCAT3M680-1 and pCAT3M680-2 indicate the same 680-bp fragment cloned in both orientations directly upstream of the CAT gene in the promoterless CAT expression vector pCAT3M. The region of the HVP *ori-P* included in the 680-bp fragment is shown (– – –).

of these constructs was equivalent to or stronger than that of the original construct shown in Fig. 4 (data not shown).

DISCUSSION

The HVP *ori-P*, first defined as the functional analog of the EBV *ori-P* (29), is shown in this work to be similar structurally to the EBV *ori-P*. Both *ori-P*s have the same general format and many special features in common on the basis of sequence. However, there are differences between the two *ori-P*s that may imply what is necessary for function.

The HVP *ori-P* consists in essence of two elements, a 5′ array consisting of 10 tandem copies of a 26-bp unit and, approximately 750 bp downstream in the genome, 5 additional imperfect and partial copies of the monomer unit. EBV *ori-P* has the same general structure, although it is larger. EBV *ori-P* has 20 tandem copies of a 30-bp monomer unit, which is related to the 26-bp HVP monomer unit. Approximately 1,000 bp downstream of this array in EBV are four additional imperfect and partial copies of the 30-bp monomer unit. Dyad structures can be proposed in this 3′ region (13), which have been hypothesized to be important in *ori-P* function (20). Similar although not identical dyad structures can be proposed for the 3′ region of the HVP *ori-P*.

A comparison of the two viral *ori-P* regions leads us to propose that the following general features are important for function. The 5′ tandem array does not need to be larger than 10 monomer units, as evidenced by the size of the HVP 5′ tandem array. This conclusion is corroborated by recent work (4, 39) in which deletion analysis of the EBV 5′ tandem array indicates that at least six or seven monomer units are needed for *ori-P* function. However, since the monomers act cooperatively rather than additively to bind EBNA-1 protein, less than a minimum number of binding sites does not simply reduce efficiency but abolishes function.

The intervening sequence between the 5′ tandem array and the 3′ array is thought to be of lesser importance in the EBV *ori-P*, because *ori-P* function is preserved in constructs in which this sequence is largely deleted. Presumably, the intervening DNA plays a minimal role in *ori-P* function by contributing to the overall structural arrangement, rather than having a direct sequence effect. The sequences of the HVP and EBV intervening DNA are quite different, except for the increasing homology (50 to 60%) of the last 400 bases approaching the 3′ dyad.

The 3′ arrays or dyad regions of EBV and HVP have four and five partial copies of the monomer units, respectively. The partial copies are homologous to each other. This region has recently been proposed to function as the site of initiation of plasmid replication in EBV (8), and we can safely say that only partial copies of the monomer unit are needed for function. The role of dyad formation is less clear, although we have shown that the area in which these structures form is spared of nucleosomes during reassembly in vitro, which may facilitate initiation of replication in latent infection (36). Both EBV and HVP have the potential to from these structures.

We have also demonstrated the existence of a transcriptional enhancer element within the *ori-P* region of HVP. Enhancement was observed in the presence of five perfect and two imperfect repeats of the monomer unit, which is the minimal number of copies required for enhancement in EBV (4, 39). In other constructs containing 10 repeats, the level of enhancement was greater. Enhancer activity was most pronounced in the HVP-infected cell line 594-S, which was expected since these cells presumably produce an authentic HVP transactivator analogous but not identical to EBNA-1. Enhancement was also observed, to a lesser degree, in the EBV-infected cell line B95-8, suggesting that the HVP enhancer can be transactivated by an EBV gene product. However, we have not identified the transactivator gene or genes, nor have we explored whether lymphoid cell factors might contribute to HVP enhancer function.

Our results clearly demonstrate that the HVP *ori-P*, like its EBV counterpart, requires transactivation by factors present in both EBV- and HVP-infected cells for both enhancer and replicative functions (29). Since binding of the EBNA-1 protein to the EBV repeat monomers appears to be inseparable from the transactivator function, and since an EBNA-1 fusion protein binds specifically to the HVP *ori-P* (C. J. Sexton, J. Griffith, and J. S. Pagano, unpublished data), it seems probable that the transactivator in HVP is an

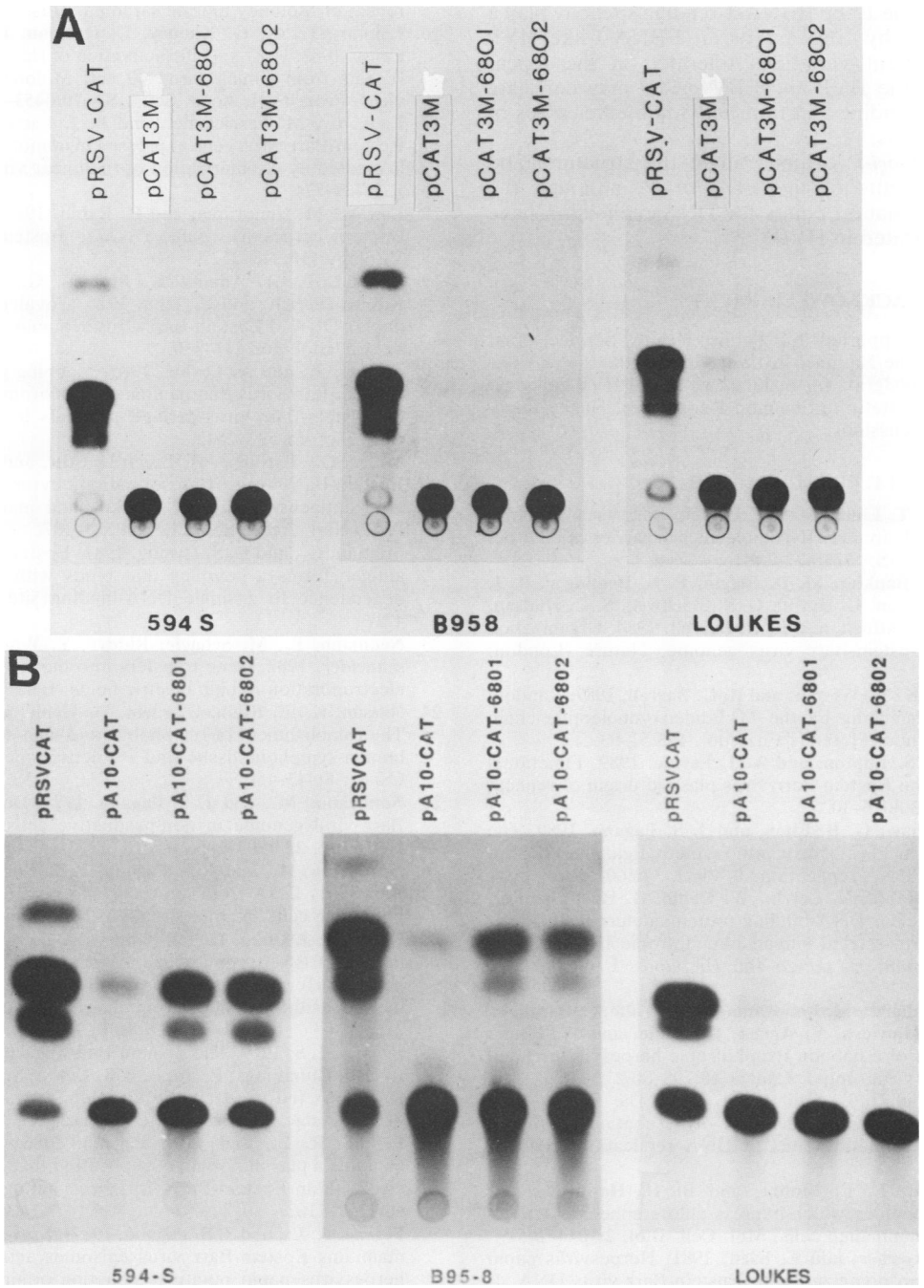

FIG. 5. HVP enhancer function shown through CAT expression in different cell lines. (A) Assay for promoter activity. pCAT3M680-1 and pCAT3M680-2 were transfected into the cell lines shown and assayed for CAT activity. (B) Assay for enhancer activity. pA_{10}CAT-680-1 and pA_{10}CAT-680-2 were transfected into the cell lines shown and assayed for CAT activity. pRSV-CAT contains the CAT gene under the control of the RSV enhancer. pA_{10}CAT contains the CAT gene under the control of the SV40 early promoter but lacks enhancer sequences.

EBNA-1-like protein. EBNA-1 is likely to be a less efficient transactivator of the HVP enhancer, and there is evidence that the EBNA-1 fusion protein binds to the HVP *ori-P* with a lower specificity (Sexton et al., unpublished data). This decrease in efficiency may be accounted for by subtle differences in the binding domains of the two proteins or by minor variations in the binding sites between the two viral *ori-P*s. Although the consensus binding sequence for EBNA-1 has been defined as a 12-bp palindrome (33), our examination of the sequence of the repeat monomers revealed an 18-bp palindromic sequence conserved between the two viruses. The 12-bp EBNA-1-binding sequence is located within this 18 bp.

In EBV, only 19 bp of the repeat monomer is conserved in both the 5′ and 3′ repeat elements. This 19-bp sequence encompasses an 18-bp palindrome conserved in EBV and HVP. Conservation of the 18-bp palindrome in both the EBV 3′ dyad and the HVP *ori-P* seems to indicate that the

sequence flanking the 12-bp EBNA-1-binding site may play a role in recognition by EBNA-1 or an EBNA-1-like HVP protein. The different variations tolerated in the repeat monomers within the HVP and EBV *ori-P*s may correlate with the different binding and transactivation efficiencies of the two viral proteins.

Thus, the HVP *ori-P* exhibits all of the structural and functional components of the EBV *ori-P*, although the initiation and termination points for DNA replication have yet to be demonstrated in HVP.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 2PO1CA19014 from the National Institutes of Health.

We thank Terri Snodgrass for making two of the HVP enhancer constructs. We are grateful to Shannon Kenney and Nancy Raab-Traub for helpful discussions.

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