Isolation and Characterization of the Genes for Two Small RNAs of Herpesvirus Papio and Their Comparison with Epstein-Barr Virus-Encoded EBER RNAs

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Genes for the Epstein-Barr virus-encoded RNAs (EBERS), two low-molecular-weight RNAs encoded by the human gammaherpesvirus Epstein-Barr virus (EBV), hybridize to two small RNAs in a baboon cell line that contains a similar virus, herpesvirus papio (HVP). The genes for the HVP RNAs (HVP-1 and HVP-2) are located together in the small unique region at the left end of the viral genome and are transcribed by RNA polymerase III in a rightward direction, similar to the EBERs. There is significant similarity between EBER1 and HVP-1 RNA, except for an insert of 22 nucleotides which increases the length of HVP-1 RNA to 190 nucleotides. There is less similarity between the sequences of EBER2 and HVP-2 RNA, but both have a length of about 170 nucleotides. The predicted secondary structure of each HVP RNA is remarkably similar to that of the respective EBER, implying that the secondary structures are important for function. Upstream from the initiation sites of all four RNA genes are several highly conserved sequences which may function in the regulation of transcription. The HVP RNAs, together with the EBERs, are highly abundant in transformed cells and are efficiently bound by the cellular La protein.

The lymphotrophic herpesviruses (Gammaherpesvirinae) (42) which infect primates can be divided into three groups; the largest group includes Epstein-Barr virus (EBV) and related viruses of Old World monkeys and apes (39). There is substantial similarity in DNA sequence (40%) and genome structure among the EBV-related viruses (12, 17, 27). All are B-cell tropic and can transform B lymphocytes in tissue culture. However, within this group, only the ability of EBV to transform cells has been studied. EBV has a genome size of 170 kilobase pairs (kb) and encodes as many as 100 gene products (3); yet only a few are expressed in transformed cells. These gene products are the EBV nuclear antigens EBNA1 to EBNA4 (23), latent membrane protein (13), and two small nonpoly(A) RNAs (EBERs) (2, 29, 43, 50). As yet, the functions of only two latent gene products have been determined. EBNA1 is involved in maintenance of the episomal viral DNA (40, 52), and the latent membrane protein is implicated in cell transformation (49).

The two EBER genes are present in one copy located 160 bases apart in the same orientation on the EBV genome (2, 43). The EBERs are RNA polymerase III transcripts of 155 to 170 nucleotides and are expressed at high levels (10^6 copies per cell) in cells transformed by EBV (2, 43). Their 3' U tails are bound by the La protein (15, 29), a 50-kilodalton protein which associates at least transiently with all RNA polymerase III transcripts in mammalian cells (14, 19, 32, 41, 46). Although the function of the EBERs is unknown, there is evidence that they can substitute for an analogous pair of RNAs (VAs) expressed by adenovirus (6, 7). The VAs prevent the interferon-induced inhibition of translation (24, 37) by interfering with the phosphorylation of a double-stranded RNA-dependent protein kinase which in turn phosphorylates protein synthesis initiation factor eIF2α. The EBERs, in contrast, have not been found to inhibit the phosphorylation of the protein kinase in vitro (20). Moreover, the subcellular location of the EBERs has been found to be nuclear (20), implying that the EBERs function in DNA replication, transcription, RNA processing, or transport.

Recently, a structural analysis revealed that the two EBERs are significantly different in their secondary structures, with several interesting features such as a single-stranded loop of 20 nucleotides in EBER2 which could be used for base pairing with other RNAs (15). To examine in more detail structural features possibly important for the functions of the EBERs, we looked for comparable RNAs in other closely related herpesviruses. Here we describe two small RNAs expressed in cell lines containing herpesvirus papio (HVP), a baboon virus whose genome is colinear with that of EBV (16, 18, 28). The cloning and sequencing of the HVP RNA genes has allowed a comparison of the secondary structures of the RNAs and suggested novel features in the transcription of these RNAs.

MATERIALS AND METHODS

Cell culture and viral DNA purification. An HVP-producing cell line obtained from E. Kieff (University of Chicago) is described elsewhere (16). Two Burkitt's lymphoma cell lines, BJAB (+EBV) and Raji (+EBV), were used for studies involving EBERs. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. To increase the level of virus produced, cells were treated with 12-O-tetradecanoylphorbol-13-acetate at 20 ng/ml for 7 days before harvesting the medium (54).

The HVP virions purified from clarified cell medium were passed through a membrane prefILTER (47). Virions were precipitated from this solution with polyethylene glycol (PEG) (1), and the DNA was purified from the isolated virus.

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the SmaI phenol-extracted lysate was ethanol precipitated, and the buffer (7 M urea, 0.35 M Tris [pH 7.9], 0.65% Nonidet P-40). The nuclease digestion proceeded for 1 h at harvest of cells, washed with water. A 598-bp polynucleotide kinase and the 3' ends were digested with restriction enzymes, and the resultant pellet was washed, dried, and suspended in water. 

To localize the 5' end of HVP-1 RNA, a 150-bp SmaI-EcoRI fragment, 5' end labeled at the SmaI restriction site, was used. A 598-bp SmaI-HindIII fragment, 3' end labeled at the SmaI site, was used to determine the 3' end of HVP-1 RNA. The 5' end of HVP-2 RNA was localized with a 463-bp SmaI-EcoRI fragment that was 5' end labeled at the 5' SmaI restriction site. To determine the 3' end of HVP-2 RNA, a 229-bp DdeI-DdeI fragment that was 3' end labeled at the 3'-most DdeI site was used. 5' ends were 32P-labeled with polynucleotide kinase and the 3' ends were 32P-labeled with Klenow fragment by the protocols of Maniatis et al. (31). S1 nuclease digestion proceeded for 1 h at 25°C, and fragments were electrophoretically separated on 8% sequencing gels. 

Hybridizations. (i) Southern blot. Purified HVP DNA was digested with restriction enzymes, and the fragments were separated on 0.4 to 1.5% agarose slab gels. The DNA fragments were transferred onto nitrocellulose as described by Southern (45) and were hybridized with HVP-1 or HVP-2 RNA isolated from gels. HVP RNAs were 5' end labeled with polynucleotide phosphorylase after dephosphorylation with calf intestine alkaline phosphatase (31).

(ii) Northern RNA blot. SP6 vectors containing genes for EBER1 and EBER2 have been described previously (20). After being digested with appropriate restriction enzymes, the vectors were transcribed by SP6 polymerase to generate 32P-labeled anti-sense RNA probes (34). RNA was extracted from cells as described above, electrophoresed onto a 10% polyacrylamide gel, and electrotransferred from GeneScreen (E. I. Du Pont de Nemours & Co., Inc.) according to the protocol of the manufacturer. The RNA blot was hybridized with 32P-labeled RNA, washed with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.01% SDS followed by 0.1× SSC, and then dried and autoradiographed.

In vitro transcription. Soluble transcription extracts were prepared from log-phase BJAB cells and stored at −70°C (11). Transcription of HVP RNA gene-containing plasmids (20 µg/ml) was carried out at 30°C for 1 h in transcription buffer (70 mM KCl, 15 mM HEPES [N-2-hydroxyethylpiperezine-N'-2-hydroxysulfonic acid] [pH 7.9], 3 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM each ATP, CTP, and UTP, 0.025 mM GTP) and 100 µCi [α-32P]GTP (410 Ci/mmol; Amer sham) per ml. Transcription was stopped with the addition of 0.4% SDS-20 µg of carrier RNA and then proteinase K (10 µg/ml) treated at 65°C for 20 min, followed by ethanol precipitation. The 32P-labeled transcripts were electrophoretically separated on 5% polyacrylamide–7 M urea gels, dried, and autoradiographed.

Immunoprecipitation. The antibodies used in immunoprecipitation were from patients with systemic lupus erythematosus (provided by J. Hardin) and were described previously (30). Immunoprecipitation was carried out with 32P-labeled cells. After sonication, the cleared lysate (107 cells) was incubated for 45 min at 4°C with anti-La or nonimmune serum (Me) prebound to swollen protein A-Sepharose (Pharmacia) in buffer A (100 mM NaCl, 2 mM MgCl2, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, 50 mM Tris [pH 7.5]). The protein A-Sepharose beads were spun down (supernatant was saved for some experiments), washed four times with buffer A, and the RNA was extracted with phenol in the presence of 0.4% SDS–20 µg of carrier RNA while being heated at 37°C for 15 min. RNAs were ethanol precipitated and separated on 5% polyacrylamide–7 M urea gels.

Computer methods. The University of Wisconsin programs were used to obtain sequence alignments and secondary structures. To align the HVP and EBV sequences, the GAP program was used. To determine the secondary structures and free energies of formation of the HVP RNAs, the FOLD program was used (53) which utilizes the base-pairing

FIG. 1. Identification of small RNAs in HVP by Northern blot analysis. RNAs isolated from BJAB (−EBV) or Raji (+ EBV) and HVP cell lines were separated on a 10% polyacrylamide–7 M urea gel. The RNA was electrotransferred to GeneScreen, hybridized with 32P-labeled SP6 antisense probes for EBER1 or EBER2, and autoradiographed. Sizes of DNA markers (in nucleotides) are indicated.
energies of Tinoco et al. (48). Regions which were previously determined to be single stranded in EBERs (15) and which were found to be conserved in the corresponding HVP RNA were constrained from base pairing.

RESULTS

Identification of EBER-like RNAs in HVP-infected cells. The significant DNA sequence similarity between EBV and an analogous virus in baboons, HVP, suggested the existence of EBER-like RNAs in HVP. In Northern blots, EBER1 and EBER2 probes were each found to hybridize to single RNA bands from HVP-containing cells (Fig. 1). No signal with either probe was observed in BJAB cells, an EBV-negative Burkitt's lymphoma cell line. The HVP-1 RNA migrates significantly slower than EBER1 from Raji cells, a Burkitt's lymphoma cell line containing EBV. The HVP-2 RNA has the same mobility as EBER2.

We next cloned a DNA fragment containing the HVP small RNA genes. This was accomplished by first isolating a large amount of HVP virus and extracting the DNA. The extracted DNA was cleaved with restriction enzymes, Southern blotted, and hybridized with 5' - end-labeled HVP in vivo RNAs which had been purified on a polyacrylamide gel. Both HVP RNAs hybridized to the EcoRI K fragment and to the XbaI-Dhet fragment of the HVP genome (Fig. 2A). The 3-kb EcoRI-XbaI fragment was isolated and further defined by cleaving it with HaeIII. A 697-bp fragment which hybridized to the HVP end-labeled small RNAs was isolated and cloned into the polylinker Small site of M13mp10. The location of the HVP RNA genes (Fig. 2B) within the small unique region at the left end of the genome and near the origin of replication (38) corresponds to the location of the EBERs within EBV DNA. This conclusion supports the colinear nature of the EBV and HVP genomes (18).

Sequence analysis of the HVP RNA genes. The sequence of the 697-bp HaeIII fragment was determined by the dideoxy sequencing method (44) (Fig. 3). The fragment contains two sets of sequences similar to the intragenic control regions of genes transcribed by RNA polymerase III (E. P. Geiduschek and G. P. Tocchini-Valentini, Annu. Rev. Biochem., in press), with downstream runs of T residues (≥4) that could act as termination signals for transcription.

To map the exact locations of the 5' and 3' ends of the HVP RNAs, we used an S1 protection assay. Two DNA fragments for each putative gene (Fig. 4A) were isolated,

FIG. 2. Organization of the HVP genome and of the DNA fragment containing the small RNA genes. (A) Partial restriction endonuclease cleavage maps of EBV DNA for enzymes EcoRI and BamHI and of HVP DNA for enzymes EcoRI and XbaI. The EBER genes and origins of replication are indicated (38, 51). (B) Enlargement of the 3-kb EcoRI-XbaI HVP DNA fragment and location of the HVP small RNA genes. Arrows show direction of transcription.

FIG. 3. Nucleotide sequence of the HVP HaeIII-HaeIII fragment containing the genes for the small RNAs. The DNA sequence of the sense strand of HVP is shown. Genes for HVP-1 and HVP-2 RNAs, outlined in boxes, were determined by S1 nuclease analysis and comparison with the EBER gene sequences. Nucleotides shown in small bold print in both genes correspond to the intragenic control regions of the RNA polymerase III promoter (Geiduschek and Tocchini-Valentini, in press). Arrows indicate the positions of 5' and 3' ends of the HVP RNAs, as determined by S1 nuclease analysis.
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4D and E). Its 5' end lies at about position 372, and its 3' end lies within the first run of T residues at bp 537 to 540. Note that the 3' ends of both HVP-1 and HVP-2 RNAs map to the position of RNA polymerase III termination signals (Geiduschek and Tocchini-Valentini, in press) in the DNA.

In vitro transcription of HVP RNA genes. To ascertain that the HVP RNAs are indeed transcribed by RNA polymerase III, we cloned the two genes onto separate plasmids and tested RNA synthesis in vitro for sensitivity to a-amanitin. Figure 5 shows resistance to low amounts (1 μg/ml) but not to high amounts (200 μg/ml) of the drug, as expected for class III genes (Geiduschek and Tocchini-Valentini, in press). Separation of the two genes also allowed identification of the slower-migrating RNA as HVP-1 RNA and of the faster-migrating RNA as HVP-2 RNA. The HVP-1 RNA gene appears to yield two RNAs, HVP-1 and HVP-1*, upon transcription in vitro. The in vivo lane also shows these two RNAs, but the slower form is much less abundant. Unfortunately, we were unable to pinpoint distinct differences between the two forms of HVP-1 RNA by S1 mapping; this was not investigated further.

HVP-1 and HVP-2 RNAs bind the La protein. Although

labeled at the relevant 3' or 5' ends (see Materials and Methods), and hybridized with in vivo RNA isolated from HVP cells or with RNA made in vitro in a Raji cell transcription extract programmed with plasmid DNA. The hybridized fragments were treated with S1 nuclease, and the S1 nuclease-resistant DNAs were separated on sequencing gels alongside corresponding DNA sequencing ladders.

The HVP-1 RNA made both in vivo and in vitro was analyzed (Fig. 4B and C). The 5'-end analysis did not produce identical protected DNA fragments for RNA from the two sources. However there was overlap at around positions 57 and 58 (Fig. 3), placing the 5' end in this region. The 3'-end analysis yielded more nearly identical fragments for the two kinds of RNA, locating the 3' end of HVP-1 RNA within the first run of thymine residues at bp 244 to 247.

HVP-2 RNA synthesized only in vivo was analyzed (Fig.

FIG. 5. In vitro transcription of the HVP small RNA genes. RNAs synthesized in a HeLa nuclear extract programmed with HVP-1, HVP-2, or HVP-1 and HVP-2 (HVP1/2) DNA were separated on a 5% polyacrylamide–7 M urea gel. Reaction experiments were performed with different amounts of α-amanitin as indicated (−, no α-amanitin). RNAs from in vivo 32P-labeled HVP cells are also shown. Sizes (in nucleotides) of RNA transcripts and DNA marker fragments are indicated.
the small RNA genes is slightly longer. HVP-2 RNA is 172 nucleotides, nearly the same length as EBER2 (169 nucleotides). The sequence similarity is 83% between HVP-1 RNA and EBER1 and 65% between HVP-2 RNA and EBER2. The regions immediately upstream of the two genes are also more highly conserved (60 and 50%) than the overall similarity between the two genomes (40%) (12, 17, 27). The distance between the two RNA genes is reduced to 133 bp in the HVP genome compared with the 160-bp distance between the two EBER genes.

The EBER and HVP small RNA genes all have intragenic control region boxes A and B, typical of RNA polymerase III type II promoters (Fig. 7B) (Geiduschek and Tocchini-Valentini, in press). The box A sequence is well conserved among the four RNA genes and matches the consensus except for the C at the third position. The box B sequence is also well conserved but shows two differences from the consensus, A residues at positions 4 and 6. The substitution at position 4 is interesting because changes at this position produce a nonfunctional promoter in Saccharomyces cerevisiae (Geiduschek and Tocchini-Valentini, in press).

The sequences upstream from the initiation sites of all four viral RNA genes, when aligned by the GAP computer program (Fig. 7C), reveal some particularly noteworthy features. Between nucleotides −20 and −28 are a series of conserved T residues, which make this region resemble the TATA boxes of RNA polymerase II promoters (9). An upstream TATA box has been found necessary for the in vitro transcription of at least one class III gene, that for 7SK RNA (35). A second region conserved in all four genes is located between positions −42 and −51 and includes the sequence TGACG, similar to the recognition site of the activating transcription factor DNA-binding protein (26). A third conserved region located between positions −61 and −67 is C rich and exhibits some similarity to the SP1 protein-binding site consensus sequence (GGGCCT) found upstream from the initiation site of various RNA polymerase II genes (22). The significance of these upstream regions has yet to be determined. However, the importance of upstream sequences for RNA polymerase III transcription has recently been underscored by studies of the 7SK gene mentioned above and of the U6 RNA gene in which a known RNA polymerase II enhancer (octamer) element has been shown to be necessary for in vivo expression (4, 10).

We used the FOLD computer program to generate secondary structures for the HVP RNAs, which are shown in Fig. 8, compared with secondary structures for the two EBERs. The EBER structures incorporate data obtained by using enzymatic and chemical probes (15); these data are
assumed to be more accurate than those based only on thermodynamic criteria. When the HVP-1 RNA structure was computed by using minor constraints that prevent base pairing, a model that resembles EBER1 very closely was obtained. Except for an insertion of about 22 bases at nucleotide 99 of EBER1, what changes there are conserve the overall secondary structure. Certain of these replacements occur in pairs (at nucleotides 2 and 182 and 46 and 57) and preserve comparable stem structures in the two viral RNAs (36).

The secondary structure of the HVP-2 RNA was initially obtained by using base-pairing constraints in the large loop of 20 nucleotides starting around position 105-106 and was found to be similar to the EBER2 structure from nucleotides 75 to 142. Nucleotides 1 to 78 and 143 to 161, however, formed a partly disrupted stem different from that of EBER2. When base pairing of the preserved C-rich region at nucleotides 75 to 78 was allowed, the resultant structure (Fig. 8B) was similar to that of EBER2, except for a longer stem from positions 20 to 65. Compensatory base changes (36) are found in the HVP-1 RNA structure at nucleotide pairs 7 and 155, 25 and 61, 28 and 58, and 32 and 54. The last three pairwise replacements occur within a stem structure which has undergone extensive base changes; the small helical segment with two base-pair changes can be considered to be proven and is defined as having two covariant base pairs within a helix (36).

The remarkable conservation of secondary structures between the HVP RNAs and the EBERs suggests that the secondary structures obtained for the EBERs by chemical and enzymatic methods are correct (36). Such conservation also implies that the RNA structures are required for function. Another example of small RNAs which have conserved their secondary structures are the VAs specified by various adenoviruses (25); they possess extensive hairpin loop structures that presumably bind the double-stranded RNA-dependent protein kinase.

It is striking that both the EBERs and the HVP small RNAs bind the La protein stably (Fig. 6). Since a large fraction of the La protein in a cell may therefore be sequestered away from other RNA polymerase III transcripts, the EBERs and HVP RNAs could serve to modulate processing events occurring at the 3′ ends of all these RNAs. In addition, a balance between La protein and small RNA production would have to be maintained so that sufficient La protein would be available to carry out its normal function within the in vivo-transformed cell.

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**FIG. 7.** Comparison of the EBV and HVP DNA sequences encoding the viral small RNAs. (A) DNA sequences of the EBV and HVP RNA genes and their flanking regions were aligned by the GAP computer program. Nucleotides which are identical are shown with a short line between them, and deletions are indicated as ( ). HVP-1 and HVP-2 RNA genes and EBER1 and EBER2 genes are boxed. Arrows indicate the locations of the termination sites for RNA transcription as determined by S1 nuclease mapping. (B) Comparison of intragenic control region boxes A and B among EBER1 and EBER2 and HVP-1 and HVP-2 RNA genes. Deletions are indicated as ( ). Circles around nucleotides indicate nonidentity among the four sequences. Consensus sequences are shown (Geiduschek and Tocchini-Valentini, in press). R, Purine; Y, pyrimidine; N, any nucleotide. (C) Comparison of 5′ flanking sequences (−1 to −67 bp) among EBER1 and EBER2 and HVP-1 and HVP-2 RNA genes. Deletions are indicated as ( ). Boxes show identities among all four DNA sequences.
FIG. 8. Secondary structures of EBERs and HVP RNAs. EBER secondary structures, as previously determined (15), were compared with HVP RNA structures whose structures and free energies of formation were determined by using the FOLD computer program of Zuker and Stiegler (53). (A) HVP-1 RNA vs. EBER1. (B) HVP-2 RNA vs. EBER2. Open rectangles represent changes in sequence between HVP and EBER RNAs as determined by the GAP computer program. Closed rectangles represent compensatory changes that conserve base pairing. The difference in free energy of formation between the unconstrained and constrained HVP-1 RNA models was −70.9 vs. −69.5 kcal (−296.6 vs. −290.8 J/mol). The free energy of formation of HVP-2 RNA constrained to be similar to the EBER2 structure only in the loop region was −48.5 kcal (−202.9 J/mol); if the additional constraint of base pairing in the C-rich region was used, the free energy of formation was −44.6 kcal (−186.6 J/mol).
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