Upstream Basal Promoter Element Important for Exclusive RNA Polymerase III Transcription of the EBER 2 Gene

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The Epstein-Barr virus-encoded small RNA (EBER) genes are transcribed by RNA polymerase III, but their transcription unit appears to contain both class II and class III promoter elements. One of these promoter elements, a TATA-like box which we call the EBER TATA box, or ETAB, is located in a position typical for a class II TATA box but contains G/C residues in the normal T/A motif and a conserved thymidine doublet. Experiments using chloramphenicol acetyltransferase constructs and mutations in the TATA box of the adenovirus major late promoter showed that the ETAB promoter element does not substitute for a class II TATA box. However, when the ETAB promoter element sequence was changed to a class II TATA box consensus sequence, the EBER 2 gene was transcribed *in vitro* by both RNA polymerases II and III. From these results, we conclude that the ETAB promoter element is important for the exclusive transcription of the EBER 2 gene by RNA polymerase III.

During the infection and immortalization of human B lymphocytes by Epstein-Barr virus (EBV), the most highly transcribed virally encoded genes are the nonpolyadenylated small RNAs called EBERs (2, 37). EBER 1 and 2, 165 and 169 nucleotides, respectively, are highly abundant (10^7 copies per cell) and bind the La protein (29, 37, 54). The EBER genes are transcribed by RNA polymerase III. Evidence for this conclusion is from data showing that their transcription is inhibited only by high amounts of α -amanitin and they terminate at a sequence of four or more thymidines. The EBER genes, however, are unusual because they have a transcription unit which contains both class II and III promoter elements (28, 54). Most class II promoters possess a TATA box (–25 to –30), which determines the start site and interacts with the transcription initiation factor TFIID. In addition, there are various numbers and combinations of usually upstream promoter elements, which bind transactivating proteins and thereby induce transcription. The three upstream class II promoter elements of the EBER genes are an Sp1 site at –65, an ATF (activating transcription factor) site at –50, and a TATA-like box at –25 to –30 (28).

Class III genes, excluding 5S, normally contain the internal control boxes A and B (references in reference 21) and require two transcription factors, TFIIB and TFIIC (56), in addition to RNA polymerase III. Determination of the start site in class III genes is directed by box A, and the level of expression is directed by box B. Sequences immediately upstream from the start site have also been found to be important for transcription of many class III genes, probably because TFIIB binds this region (31, 32). Although a consensus sequence has not been found, many of these upstream regions contain TATA-like sequences. The class III promoter elements of the EBER genes are functionally similar to the internal control boxes A and B (28, 54). Therefore, the EBER genes appear to be typical class III

genes with an upstream set of promoter elements normally associated with class II genes.

Recently, other unusual class III genes have been described. Members of this group, containing the U6 and 7SK genes, deviate from normal class III gene transcription units by not having functional internal class III promoter elements (3, 34, 43, 51; reviewed in references 34 and 44). Some of the promoter elements of the U6 and 7SK genes are also normally found associated with other U small nuclear RNA (snRNA) genes, which are believed to be transcribed by RNA polymerase II. These are the distal sequence element, which contains an octamer motif, and proximal sequence element, which determines the transcriptional start site and is functionally similar to the class II TATA box (16). However, the U6 and 7SK genes are transcribed by RNA polymerase III. In the U6 gene, another promoter element, not found in other U snRNA genes, functions to determine RNA polymerase III transcription of the U6 gene (38, 41). This promoter element is surprisingly similar in sequence and location to the class II TATA box. When the U6 TATA-like promoter element is placed at the same location in the U2 gene, the U2 gene is transcribed by RNA polymerase III (38). This incongruous result suggests that the class II TATA box can direct the RNA polymerase III transcription of a normal class II U snRNA gene.

The class II TATA box is normally located at –25 to –30 and functions to determine the transcriptional start site of most protein-coding genes (5). The TATA box consensus sequence is 5'-TATAAA-3' and does not tolerate G/C substitutions (15, 45, 60). The TATA box binds an important RNA polymerase II initiation factor, TATA-binding protein (TBP), and its binding is considered the first step in the initiation of transcription by RNA polymerase II (10, 17, 20, 46, 47, 52). The purification of TBP has been difficult, but the yeast equivalent has been cloned and the protein has been purified to homogeneity (12, 19, 23, 26, 55) and found to functionally substitute for the human TBP (11, 13). The yeast and recently cloned human TBPs (25, 30) are small, single-subunit proteins that are unable to perform all activities of the partially purified mammalian TBP, such as response to upstream promoter elements (49). The partially purified

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mammalian TBP is larger than the purified TBP core protein, suggesting that the mammalian TBP occurs naturally in multimeric protein complexes containing possible coactivators, which function with the upstream promoter elements (11, 49, 52). Until recently, TBP had been found to function only with class II genes. However, investigations of the yeast and vertebrate U6 genes, which at least in the case of the vertebrate U6 gene contain a functional TATA-like box, show that TBP is required for in vitro RNA polymerase III transcription (39, 40, 57). In addition, recent evidence suggests that TBP is a ubiquitous transcription factor important not only for class III gene transcription (59) but also for transcription of class I genes (14).

The observations that the EBER genes contain complete sets of both RNA polymerase II and III promoter elements yet are transcribed by RNA polymerase III prompted us to question why they are not transcribed by RNA polymerase II. This study investigates more thoroughly the TATA-like box located at -25 to -30, which we call the EBER TATA box (ETAB) promoter element. Previous work has shown that a mutation in the ETAB promoter element decreases EBER transcription (28). Although the ETAB promoter element is located at a class II TATA box position, it does not conform to the consensus sequence of the class II TATA box because it contains G nucleotides in the otherwise normal T/A motif. In addition, there is a thymidine doublet conserved in four viral small RNA gene TATA boxes at the same position relative to the beginning of the ETAB promoter element. We show that the ETAB promoter element plays an important role in the exclusive RNA polymerase III transcription of the EBER 2 gene.

MATERIALS AND METHODS

Cell culture and materials. Cell lines BJAB, Raji, and HeLa were grown in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer by John Flory. α -Amanitin was purchased from Sigma Chemical Co.

Mutant constructions. (i) **EBER 2 (E2)/WT.** Construct D in reference 28.

(ii) **E2/-43.** Construct F in reference 28.

(iii) **E2/-8.** Construct G in reference 28.

(iv) **E2/Sp1.** Construct H in reference 28.

(v) **E2/ATF.** Construct I in reference 28.

(vi) **E2/TATA.** Construct J in reference 28.

(vii) **E2/(-A).** Construct K in reference 28.

(viii) **E2/(-B).** Construct L in reference 28.

(ix) **E2/G.** The oligonucleotide 5'-CGAACCGTAATTTT ATACATGC-3' was used to create a double-point mutation in the region -28 TATAGAGTT -20, which converts it to -28 TATAAAATT -20 upstream of the EBER 2 gene in the E2/WT construct (28, 35).

(x) **E2/T.** The oligonucleotide 5'-CGAACCGTCCCTCTA TACATGC-3' was used to create a double-point mutation in the region -28 TATAGAGTT -20, which converts it to -28 TATAGAGGG -20 upstream of the EBER 2 gene in the E2/WT construct (28).

(xi) **E2/GT.** The oligonucleotide 5'-CGAACCGTCCTTT TATACATGC-3' was used to create a quadruple-point mutation in the region -28 TATAGAGTT -20, which converts it to -28 TATAAAAGG -20 upstream of the EBER 2 gene in the E2/WT construct (28).

(xii) **E2/TATA(G).** The E2/G mutation was introduced into E2/TATA as described in paragraph ix.

(xiii) **E2/GT(-A).** The E2/GT mutation was introduced into E2/(-A) as described in paragraph xi.

(xiv) **E2/GT(-B).** The E2/(-B) mutation was introduced into E2/GT by digesting E2/GT with *Ava*I, filling the ends with Klenow polymerase, and religating.

(xv) **E2/GT(-A,-B).** The E2/(-B) mutation was introduced into E2/GT(-A) as described in paragraph xiv.

(xvi) **E2/WT-CAT.** The E2/WT plasmid described in paragraph i was digested with *Hind*III and *Eco*RI, and the 547-bp fragment was isolated. This *Hind*III-*Eco*RI fragment was ligated with *Hind*III-*Eco*RI-digested M13mp19. By using the oligonucleotide 5'-CGGCTGTCCGGTTTGATGTAGC-3', an *Hpa*II restriction site was introduced by oligonucleotide mutagenesis at the +1 position of the EBER 2 gene. After mutagenesis, the DNA was digested with *Sma*I and *Hind*III, the 265-bp fragment was isolated and further digested with *Hpa*II, and 3' end was filled with Klenow polymerase. The DNA was then ligated with pSV2CAT digested with *Hind*III and *Acc*I and 3' end filled with Klenow polymerase. The orientation was determined, and the plasmid with the fragment inserted so that the *Hpa*II site closest to the chloramphenicol acetyltransferase (CAT) gene was selected.

(xvii) **E2/WT reversed-CAT.** Same as E2/WT-CAT except that the fragment was in the reverse or opposite orientation, with the *Hpa*II site furthest away from the CAT gene.

(xviii) **E2/G-CAT.** The E2/G mutation was introduced into E2/WT-CAT as described in paragraph ix.

(xix) **E2/T-CAT.** The E2/T mutation was introduced into E2/WT-CAT as described in paragraph x.

(xx) **E2/GT-CAT.** The E2/GT mutation was introduced into E2/WT-CAT as described in paragraph xi.

(xxi) **ML/WT.** The *Bam*HI-*Sac*I fragment containing the adenovirus type 2 major late promoter (MLP) contiguous sequences (5774 [-375] to 6233 [+99]; 7012 to 7602; 9271 to 9300) was ligated to *Bam*HI-*Sac*I-digested pGEM3Z. The adenovirus major late sequence DNA was kindly provided by N. Hernandez.

(xxii) **ML/GGTT.** The ML/GGTT mutation was introduced into ML/WT, using the oligonucleotide 5'-CGCGCCCCAC CCAACTCTATAGCCCCC-3' to create a quadruple-point mutation in the region -33 GCTATAAAAGGGG -21, which converts it to -33 GCTATAGAGTTGG -21 upstream of the adenovirus MLP start site.

(xxiii) **ML/GTT.** The ML/GTT mutation was introduced into ML/WT, using the oligonucleotide 5'-CGCGCCCCAC CCAACTTTATAGCCCCC-3' to create a triple-point mutation in the region -33 GCTATAAAAGGGC -21, which converts it to -33 GCTATAAAGTTGC -21 upstream of the adenovirus MLP start site.

(xxiv) **ML/G.** The ML/G mutation was introduced into ML/WT using the oligonucleotide 5'-CGCGCCCCACCCC CCTCTATAGCCCCC-3' to create a single-point mutation in the region -33 GCTATAAAAGGGC -21, which converts it to -33 GCTATAGAAGGGC -21 upstream of the adenovirus MLP start site.

(xxv) **E2/ATF/TATA(G).** The E2/ATF construct described in paragraph v was digested with *Bam*HI and *Alu*I, and the 115-bp fragment containing the EBER 2 sequences (-43 to -158) was isolated. The E2/TATA(G) construct described in paragraph xii was digested with *Alu*I and *Eco*RI, and the 402-bp fragment containing the EBER sequences (-43 to +359) was isolated. These two fragments were combined with *Bam*HI-*Eco*RI-digested pGEM3Z DNA and ligated. The resultant construct contained the E2/ATF and E2/TATA(G) mutations.

(xxvi) **E2/Sp1/TATA(G).** The E2/Sp1 construct described

in paragraph iv was digested with *Hind*III and *Alu*I, and the 145-bp fragment containing the EBER 2 sequences (–43 to –158) was isolated. The E2/TATA(G) construct described in paragraph xii was digested with *Alu*I and *Eco*RI, and the 402-bp fragment containing the EBER sequences (–43 to +359) was isolated. These two fragments were combined with *Hind*III-*Eco*RI-digested pGEM3Z DNA and ligated. The resultant construct contained the E2/Sp1 and E2/TATA(G) mutations.

(xxvii) **E2/TATA(G) #2.** The E2/WT construct described in paragraph i was digested with *Bam*HI and *Alu*I, and the 115-bp fragment containing the EBER 2 sequences (–43 to –158) was isolated. The E2/TATA(G) construct was digested with *Alu*I and *Eco*RI, and the 402-bp fragment containing the EBER sequences (–43 to +359) was isolated. These two fragments were combined with *Bam*HI-*Eco*RI-digested pGEM3Z DNA and ligated. The resultant construct contained the E2/TATA(G) mutation.

Transfections and RNase protection. Transfection experiments were done by using BJAB cells and 2 µg of added DNA as described previously (28). The transcription products were assayed by RNase protection by using an EBER 2 antisense probe and electrophoresing the products onto a 6% polyacrylamide–7 M urea gel as described previously (28). Quantitation was performed with a Joyce-Loebl densitometer.

In vitro transcription. Transcription extracts were prepared from BJAB, Raji, and HeLa cells according to the method of Dignam et al. (18). Transcription of the EBER gene-containing plasmids (0.8 µg/ml, plus 20 µg of carrier pGEM3Z plasmid per ml) and adenovirus MLP-containing plasmids (40 µg/ml) was carried out with either linearized (RNA polymerase II) or intact (RNA polymerase III) DNA. The reaction mixtures were incubated at 30°C for 1 h in transcription buffer (70 mM KCl, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 3 mM MgCl₂ [RNA polymerase III] or 12 mM MgCl₂ [RNA polymerase II], 0.5 mM dithiothreitol, 0.5 mM each ATP, CTP, and UTP, 0.025 mM GTP) and 100 µCi of [α -³²P]GTP (410 Ci/mmol; Amersham) per ml. Cell extract was included to give a final volume of 25 µl. In some reaction mixtures, α -amanitin was added at 1 µg/ml (inhibits RNA polymerase II) or 100 µg/ml (inhibits RNA polymerase III). Transcription was stopped with the addition of 0.4% sodium dodecyl sulfate and 20 µg of carrier tRNA; the mixture was then proteinase K (10 µg/ml) treated at 65°C for 20 min and ethanol precipitated. The ³²P-labeled transcripts were electrophoretically separated on 6% polyacrylamide–7 M urea gels, dried, and autoradiographed. Quantitation was performed with a Joyce-Loebl densitometer.

CAT assays. Plasmid DNA containing CAT constructs (2 µg) was transfected into Raji cells by the DEAE-dextran method as described previously (28). After 48 h, the cells were extracted and analyzed for CAT expression according to Gorman et al. (22). The experiment was done in duplicate. Quantitation was performed with a Joyce-Loebl densitometer.

Primer extension. Cold in vitro-transcribed RNA was isolated after transcription of the construct DNA. [γ -³²P]ATP-kinased primer oligonucleotide was hybridized to the cold RNA and then extended by using reverse transcriptase. The extension products were applied to a 10% polyacrylamide–7 M urea gel.

RESULTS

The ETAB promoter element does not function as a class II TATA box. Previously we showed that the region –33 to –20 upstream of the EBER 1 and 2 and herpesvirus papio HVP1 and HPV2 RNA genes is highly conserved and resembles a class II TATA box consensus sequence (Fig. 1A [27, 28]). The conserved nucleotides of these viral TATA-like boxes, which we call ETAB promoter elements, are T nucleotides at positions corresponding to the first and third nucleotides of the consensus sequence of the class II TATA box (Fig. 1A [5]). In addition, there are G nucleotides dispersed throughout the ETAB promoter element sequences located primarily at positions 4, 5, and 6 of the class II TATA box consensus sequence (Fig. 1A), which are nearly always T/A base pairs (5). Previous studies have shown that changes in the class II TATA box sequence inhibit transcription (15, 45, 60). Specifically, guanine nucleotides replacing adenine nucleotides in positions 4 and 5 of the class II TATA box have consistent and substantial effects on the level of transcription (1). Therefore, we hypothesized that the unusual ETAB promoter element does not function as a class II TATA box but allows the exclusive transcription of the viral genes by RNA polymerase III.

Initially we tested whether the ETAB promoter element could function as a normal class II TATA box. The upstream EBER 2 gene sequence from –1 to –120 was inserted in a normal and a reversed orientation upstream from the bacterial CAT gene at a *Hind*III site (Fig. 1B). These constructs along with a simian virus 40 control were transfected into Raji cells (EBV-containing Burkitt's lymphoma cell line), and CAT expression was assayed as described in Materials and Methods. Expression from an upstream sequence containing the wild-type ETAB promoter element in the normal orientation was slightly higher than in the no-extract control (Fig. 1B; compare lanes 1 and 2), but this level was not different from that of the reverse configuration (compare lanes 2 and 3). We conclude that there was no significant CAT expression when the wild-type ETAB promoter element was present.

To test specifically the effect of the ETAB promoter element sequence on RNA polymerase II transcription, the class II TATA box of the adenovirus MLP was changed to the ETAB promoter element sequence. In vitro transcription of the adenovirus MLP constructs showed that the level of the 1,183-nucleotide runoff transcript (Fig. 1C, arrow) generated by RNA polymerase II transcription of the adenovirus MLP was reduced 10-fold when the ETAB promoter element sequence (Fig. 1A; ML/GGTT construct) was present (Fig. 1C; compare lanes 1 and 2). In vitro runoff transcription of both the ML/WT and ML/GGTT constructs was sensitive to low amounts of α -amanitin and was therefore due to RNA polymerase II (compare lanes 1 and 2 with lanes 5 and 6). From these data, we conclude that the ETAB promoter element does not substitute for a class II TATA box for transcription by RNA polymerase II.

Can the ETAB promoter element sequence be changed so that it can function as a class II TATA box? The ETAB promoter element in the E2/WT-CAT construct was mutated to a sequence similar to the consensus class II TATA box sequence, in which both the G nucleotides and T doublet were changed (Fig. 1B; E2/GT-CAT construct). The level of expression from this construct was sixfold above background (Fig. 1B; compare lanes 2 and 6) but lower than that of the simian virus 40 control (Fig. 1B; compare lanes 6 and 7). Two additional constructs, E2/G-CAT, which changed

FIG. 1. (A) TATA box regions of various constructs. See Materials and Methods for details of construction. The sequences were aligned relative to the first T nucleotide of the RNA polymerase II TATA box consensus sequence. The boxes surround the region of the EBER 2 and adenovirus MLP upstream sequences which were

only the G nucleotides, and E2/T-CAT, which changed only the T doublet, were also made (Fig. 1A). Their levels were similar (Fig. 1B, lanes 4 and 5) but lower than that of the E2/GT-CAT construct (lane 6). Similar but not identical mutations were incorporated into the adenovirus MLP TATA box (Fig. 1A; constructs ML/GTT and ML/G). In vitro transcription of these adenovirus MLP constructs showed that substituting the A nucleotide at position -27 for a G nucleotide was all that was necessary to reduce by fivefold the level of expression (Fig. 1C, lane 4). Mutation of the conserved thymidine doublet had little effect (60% of normal) on adenovirus MLP expression (Fig. 1C, lane 3), a result different from that seen with the E2/T-CAT construct (Fig. 1B, lane 5).

Another way to examine whether the ETAB promoter element functions mechanistically like a class II TATA box is to determine the effect on EBER transcription of changing the ETAB sequence to the optimal TATA box consensus sequence. The three mutations (E2/G, E2/T, and E2/GT) were incorporated into the EBER 2 ETAB promoter element upstream of a wild-type EBER 2 gene and were transfected into BJAB cells (Burkitt's lymphoma cell line not containing EBV). The level of EBER 2 expression was analyzed by a RNase protection assay as specified in Materials and Methods. The E2/G and E2/GT constructs reduced EBER 2 expression by 10- to 15-fold, whereas the E2/T construct had little effect (Fig. 2A; compare lanes 6 to 8 with lane 2). This experiment was done in triplicate with the same result. Thus, mutating the two G nucleotides inhibited EBER 2 transcription, while mutating the thymidine doublet had no effect.

The results presented above demonstrate that the ETAB promoter element is not a functional class II TATA box and in fact, when changed to a class II TATA box, inhibits EBER 2 transcription. The critical nucleotides within the ETAB promoter element for EBER 2 transcription appear to be the G nucleotides, not the thymidine doublet.

The ETAB promoter element is important for EBER 2 transcription. Previous work has shown that a mutation in the ETAB promoter element affects the transcription of the EBER 2 gene (28). This mutation, E2/TATA, reduced EBER 2 transcription by 15-fold (Fig. 2A; compare lanes 2 and 3). To further establish the importance of the ETAB promoter

mutated. Underlining designates changed nucleotides in the various constructs. The Pol II TATA sequence is the RNA polymerase II TATA box consensus sequence as determined previously (5), in which T-1 appears at position -27 to -33 relative to the start site. HVP1 and HVP2 are small RNAs encoded by herpesvirus papio (27). (B) Transcription of E2-CAT constructs in Raji cells. A schematic representation of the constructs containing ETAB mutant sequences is shown at the top. The constructs and CAT assay method are described in Materials and Methods. Construct EBER WT reverse contains the -1 to -120 sequence fragment in reversed orientation. In lane 7 (simian virus 40 [SV40]), the pSV2CAT construct (22) was used. The CAT assay was done in duplicate with similar results. Percent conversion is shown. (C) In vitro transcription of adenovirus MLP constructs. Constructs used are described in Materials and Methods, and TATA box nucleotide changes are depicted in panel A. In vitro transcription with *Eco*RI-linearized DNA at 1 μ g per reaction and containing 12 mM Mg^{2+} is described in Materials and Methods. The resultant runoff transcript of 1,183 nucleotides (nt) is indicated by an arrow. Markers (M) are *Hpa*II-digested pBR322. Quantitation of the runoff transcript determined levels for lane 1 (100%), lane 2 (11%), lane 3 (62%), and lane 4 (21%). There are many additional bands in some of the lanes which are either degradation or premature stops by RNA polymerase II.

FIG. 2. (A) Transcription of EBER 2 constructs in BJAB cells. Constructs are described in Materials and Methods. DNA (2 μ g) was transfected into BJAB cells, and the EBER 2 RNA (arrow; 170 nucleotides [nt]) was analyzed by an RNase protection assay. The marker lane (M) is as for Fig. 1C. Quantitation of the EBER transcript signals showed levels for lane 2 (100%), lane 3 (7%), lane 4 (52%), lane 5 (30%), lane 6 (6.4%), lane 7 (83%), lane 8 (8.8%), lane 9 (1.3%), lane 10 (0.7%), and lane 11 (0.8%). (B) In vitro RNA polymerase III transcription of E2 constructs. Constructs and the in vitro transcription assay using Raji nuclear extract are described in Materials and Methods. Quantitation of the EBER transcript signal showed levels for lane 1 (100%), lane 2 (4.8%), lane 3 (3.7%), lane 4 (1.5%), lane 5 (2.2%), lane 6 (6.2%), lane 7 (12.1%), lane 8 (1.3%), and lane 9 (0.4%).

element for EBER 2 transcription, additional constructs were made in which the E2/TATA and E2/G mutations were combined to form the E2/TATA(G) construct (Fig. 1A), and this construct was paired with the E2/ATF and E2/Sp1 mutations to form the E2/ATF/TATA(G) and E2/Sp1/TATA(G) constructs. When the E2/TATA(G) construct was transfected into BJAB cells, it was transcribed at a very low level (about 1% of the level of E2/WT) (Fig. 2A, lane 9). The level of expression of the E2/TATA(G) construct was more similar to the levels of constructs E2/-43 and E2/-8, which delete most of the upstream EBER 2 sequence (Fig. 2A, lanes 10 [E2/-43] and 11 [E2/-8]). Both the E2/TATA and E2/TATA(G) constructs expressed the EBER 2 gene at levels below that of constructs E2/ATF and E2/Sp1 containing mutations in the other two upstream promoter elements (Fig. 2A; compare lane 9 with lanes 4 [E2/ATF] and 5 [E2/Sp1]). This result suggests that the ETAB promoter element is necessary for transcription of the EBER 2 gene. Further support for the importance of the ETAB promoter element in EBER transcription is shown in Fig. 2B. E2 constructs transcribed in an in vitro transcription assay were expressed at levels similar to those found in the in vivo assay, although the levels of ATF and Sp1 constructs were lower in the in vitro assay. These constructs were nonetheless transcribed at the same or higher level than were the E2/TATA and E2/TATA(G) constructs (compare various constructs transcribed in Fig. 2). The levels of expression in the in vitro transcription assay were dependent only on the upstream promoter elements when low amounts of specific DNA (25 ng per assay) were added along with carrier DNA (250 ng per assay). High levels of specific DNA showed no response to upstream promoter elements, which explains why in previous work no dependence on the upstream sequence was seen (28). In Fig. 2B, the E2/TATA(G), E2/ATF/TATA(G), and E2/Sp1/TATA(G) constructs were

expressed at similar levels (Fig. 2B; compare lanes 2, 3, 4, and 5) that were much lower (<5%) than that of the E2/WT construct and also lower than that of the E2/Sp1 construct and similar to that of the E2/ATF construct (Fig. 2B; compare lanes 2, 3, 4, and 5 with lanes 1, 6, and 7). As a further control, an additional construct [E2/TATA(G) #2], which had the same amount of upstream EBER DNA sequence (-1 to -158) as did the E2/ATF/TATA(G) and E2/Sp1/TATA(G) constructs, was assayed and found to have a similar low level of expression (Fig. 2B, lane 5). This experiment shows that the added ATF and Sp1 mutations did not further reduce EBER 2 expression compared with the E2/TATA(G) mutation alone. We conclude from these results that the ETAB promoter element is necessary for full expression of the EBER 2 gene.

The ETAB promoter element prevents RNA polymerase II transcription of the EBER 2 gene. Up to this point, we have shown that the ETAB promoter element is important for EBER 2 transcription but does not function as a class II TATA box. Might it therefore act to exclude RNA polymerase II from transcribing the EBER 2 gene? This possibility was examined by determining the level of in vitro RNA polymerase II and III transcription of the E2/GT construct, which changes the ETAB promoter element sequence to a canonical class II TATA box sequence. We found that by changing the magnesium concentration of the in vitro transcription assay, optimum levels of RNA polymerase II (12 mM Mg^{2+}) or III (3 mM Mg^{2+}) transcription could be obtained. In vitro transcription under RNA polymerase II conditions (12 mM Mg^{2+}) of the wild-type EBER 2 gene linearized with *EcoRI* showed no transcription product at the expected runoff transcript length of 360 nucleotides (Fig. 3A, lane 1; designated Pol II runoff transcript). In contrast, transcription of the E2/GT construct showed a high level of the 360-nucleotide runoff transcript (Fig. 3A, lane 3). The transcription seen in lane 3 was directed by RNA polymerase II because it was inhibited by low levels of α -amanitin (Fig. 3A, lane 7). The E2/GT construct was transcribed by RNA polymerase III even at the high Mg^{2+} concentration, because a band in lane 7 of the correct length (170 nucleotides; designated Pol III) was sensitive only to high levels of α -amanitin (Fig. 3A; compare lanes 3, 7, and 11).

To rule out the possibility that runoff transcription from the E2/GT construct by RNA polymerase II was initiating elsewhere in the plasmid, primer extension analysis was performed on in vitro-transcribed RNA. A DNA primer complementary to nucleotides +33 to +50 of the EBER 2 gene was used to analyze in vitro transcripts made under RNA polymerase II and III conditions. Figure 3B shows that the 5' ends of RNA polymerase II transcripts were primarily at the +3 and +4 positions of the EBER 2 gene, compared with the +1 start for the RNA polymerase III transcripts (compare lanes 1 and 3). Addition of low amounts of α -amanitin (Fig. 3B, lane 2) revealed that most of the transcripts in lane 3 were generated by RNA polymerase II.

To determine which base changes were necessary for RNA polymerase II to transcribe the EBER 2 gene, constructs E2/G, E2/T, and E2/GT were examined under RNA polymerase II transcription conditions in vitro. The 360-nucleotide RNA polymerase II runoff transcript (Fig. 3C; designated Pol II runoff transcript) was seen only with the E2/GT construct, demonstrating that changing not only the G nucleotides but also the conserved thymidine doublet is necessary (Fig. 3C; compare lanes 2, 3, and 4). Low amounts of α -amanitin completely eliminated the appearance of the RNA polymerase II runoff transcript with the E2/GT con-

struct (Fig. 3C; compare lanes 4 and 7), proving that the runoff transcript, which is the correct size, was generated by RNA polymerase II. This result was similar to that in Fig. 1B; i.e., the E2/G-CAT and E2/T-CAT constructs had lower levels of expression than did the E2/GT-CAT construct. However, the result was different than that found with the adenovirus MLP TATA box constructs in which the introduction of only one G nucleotide reduced RNA polymerase II transcription. The EBER genes may be unique in that the thymidine doublet further safeguards them from transcription by RNA polymerase II.

RNA polymerase III transcription of the U6 gene requires a class II TATA box sequence. Without it, the U6 gene is transcribed by RNA polymerase II in addition to being transcribed weakly by RNA polymerase III (38). In the case of the EBER genes, could the opposite be occurring? Is the presence of the ETAB promoter element absolutely required for exclusive RNA polymerase III transcription of the EBER 2 gene, while without it the EBER 2 gene would be transcribed by RNA polymerase II? This question was addressed in the experiment shown in Fig. 3D in which the E2/GT and E2/TATA(G) constructs were transcribed in vitro under RNA polymerase II and RNA polymerase III conditions. The results show that under RNA polymerase III conditions, both the E2/GT and E2/TATA(G) constructs were transcribed by RNA polymerase III because only high amounts of α -amanitin inhibited transcription (Fig. 3D, lanes 1 to 6). Conversely, under RNA polymerase II conditions, only the E2/GT construct was transcribed by RNA polymerase II, as evidenced by its low α -amanitin sensitivity (Fig. 3D, lanes 7 to 12). Therefore, the ETAB promoter element is not absolutely required for the exclusive transcription of the EBER 2 gene by RNA polymerase III because even in its absence [the E2 TATA(G) construct], the EBER 2 gene was transcribed by RNA polymerase III.

It was surprising that the EBER 2 gene could be transcribed by RNA polymerase II with the class III internal control boxes A and B present. When box B was removed, the level of RNA polymerase II expression remained unchanged (Fig. 3A, lane 4). This result was also seen with the E2/GT(-A) construct (results not shown). Deletion of both boxes A and B [E2/GT(-A,-B)] likewise did not increase the RNA polymerase II transcription of the EBER 2 gene (Fig. 3C, lane 5). We conclude that the presence of these internal RNA polymerase III promoter elements is not deleterious to RNA polymerase II transcription of the EBER 2 gene, although we have not demonstrated that these sites are occupied to a large extent on the majority of DNA templates under the transcription conditions used in these assays.

The results presented above show that the EBER 2 gene can be transcribed by RNA polymerase II if the G nucleotides as well as the thymidine doublet are changed to make

the ETAB promoter element resemble a consensus class II TATA box sequence. We conclude that the ETAB promoter element functions in part to exclude RNA polymerase II from transcribing the EBER 2 gene.

DISCUSSION

What is the function of the ETAB promoter element? We have characterized a novel promoter element (ETAB) located upstream from the EBER 2 gene start site. The ETAB promoter element is similar in sequence and location to the class II TATA box but surprisingly does not functionally substitute for a class II TATA box (Fig. 1). Nonetheless, the ETAB promoter element is necessary for EBER 2 transcription, since mutations in the ETAB promoter element drastically decrease EBER 2 transcription both in vivo and in vitro (Fig. 2). Because of these observations and the unusual combination of class II and III promoter elements in EBER genes, we hypothesized five possible functions for the ETAB promoter element. First, the ETAB promoter element could enhance transcription in a manner similar to that of the nearby Sp1 or ATF upstream promoter elements. Second, it could be an RNA polymerase III determinant as demonstrated for the class II TATA box when placed in the setting of a U snRNA gene (38, 41). Third, it could act as a bridge or adaptor to allow the upstream RNA polymerase II promoter elements (ATF and Sp1) to interact with RNA polymerase III transcriptional machinery. Fourth, it could allow exclusive RNA polymerase III transcription of the EBER genes by preventing competition with RNA polymerase II for the proteins binding to the TATA box site and the upstream promoter elements. Fifth, the ETAB promoter element could be an important binding site for the basal RNA polymerase III transcription factors similar to the box A and B internal elements.

Considering the first hypothesis, we observed in Fig. 2 that when the ETAB promoter element was the only upstream promoter element (construct E2/-43), in vivo and in vitro EBER 2 expression was much lower than E2/WT expression and more similar to expression of the E2/-8 construct, in which all relevant upstream sequences were deleted (compare lanes 2, 10, and 11 in Fig. 2A and lanes 1, 8, and 9 in Fig. 2B). Therefore, the ETAB promoter element does not by itself dramatically increase transcription of the EBER 2 gene, but it may affect transcription if substituted for an upstream promoter element in another gene.

The second hypothesis has not been tested. However, when we mutated the wild-type ETAB promoter element to E2/TATA(G), which no longer resembles any known TATA box sequence, the type of polymerase transcribing the gene was not changed; only the level of RNA polymerase III transcription was reduced (Fig. 3D). Thus, the ETAB pro-

FIG. 3. (A) In vitro transcription of EBER 2 constructs. Constructs are described in Materials and Methods. In vitro transcription with *EcoRI-HindIII*-digested DNA at 0.02 μ g per reaction and including 12 mM Mg^{2+} with added carrier DNA (0.5 μ g; pGEM3Z) is described in Materials and Methods. Pol II runoff transcript designates the RNA polymerase II transcript of 360 nucleotides (nt). Pol III designates the RNA polymerase III transcript of 170 nucleotides. The marker lane (M) is as for Fig. 1C. There are many additional bands in some of the lanes which are either degradation or premature stops of RNA polymerase II. (B) In vitro transcription of EBER 2 constructs. Unlabeled RNA was isolated from an in vitro transcription assay, using construct DNA at 0.25 μ g per reaction. The reaction in lane 1 contained intact DNA and 3 mM Mg^{2+} . In lanes 2 and 3, the reactions included DNA digested with *EcoRI* and *HindIII* and 12 mM Mg^{2+} . A [γ - 32 P]ATP-kinased oligonucleotide (start at +33) was used as the primer for extension by reverse transcriptase. The extension products were applied to a 10% polyacrylamide-7 M urea gel. In some reactions, α -amanitin was added at 1 μ g/ml. (C) In vitro RNA polymerase II transcription of EBER 2 constructs as described for panel A. (D) In vitro transcription of EBER 2 constructs. Constructs are described in Materials and Methods. RNA polymerase III transcription assays in lanes 1 to 6 contained 3 mM Mg^{2+} and circular plasmid DNA. In vitro RNA polymerase II transcription assays in lanes 7 to 12 are as described for panel A. In some reactions, α -amanitin was added at 1 or 100 μ g/ml.

motor element is not absolutely required for the RNA polymerase III transcription of the EBER gene. To obtain RNA polymerase II transcription of the EBER gene, a class II TATA box sequence needs to be present.

The fourth hypothesis stems from the initial incongruous result that the EBER genes, which appear to possess a complete RNA polymerase II promoter (ATF, Sp1, and TATA box), are not transcribed by RNA polymerase II. We have shown the ETAB promoter element, which resembles but is not identical in sequence to a class II TATA box, does not function as one. Surprisingly, when the ETAB promoter element sequence was changed to a class II TATA box consensus sequence, the EBER 2 gene was transcribed by both RNA polymerases II and III (Fig. 3A). This result suggests that the ETAB promoter element is the only structure preventing the EBER 2 gene from being transcribed by RNA polymerase II. However, RNA polymerase II transcription of the EBER gene requires the introduction of a class II TATA box rather than simply mutation of the ETAB promoter element. The E2/TATA(G) mutation, which completely changes the ETAB promoter element, is not transcribed by RNA polymerase II. Therefore, although the ETAB promoter element can be converted so that the EBER gene is transcribed by RNA polymerase II, it is not absolutely required to prevent RNA polymerase II transcription of the EBER gene.

The third hypothesis and the fifth hypothesis, which states that the ETAB promoter element is important for binding by the basal RNA polymerase III transcription machinery, cannot be tested until it is known which proteins bind to the ETAB promoter element.

From the foregoing considerations, we conclude that the ETAB promoter element is present to permit high expression of the EBER 2 gene by RNA polymerase III. Its function may be to allow the other upstream promoter elements, ATF and Sp1, to stimulate RNA polymerase III transcription of the EBER 2 gene without the interference of RNA polymerase II.

How does the function of the ETAB promoter element compare with those of upstream regions of other class III genes? The low level of expression of the E2/TATA(G) construct is interesting because it reveals the inadequacy of boxes A and B alone to promote transcription of the EBER 2 gene. There are many examples of other class III genes, such as 5S and tRNA genes, which require upstream sequences for transcription (references in reference 21). These upstream regions do not necessarily have a consensus sequence, but many, like the ETAB promoter element, resemble a class II TATA box in location and sequence. What distinguishes the ETAB from these other class III elements is its necessity to function with other upstream promoter elements; therefore, its sequence requirements are probably more stringent. For example, our data show that the ETAB promoter element sequence cannot be changed to a class II TATA box, because the EBER 2 gene is then transcribed by RNA polymerase II. By analogy, a 5S or tRNA promoter which has upstream sequences resembling a class II TATA box (such as the 5S RNA and alanine tRNA genes of *Bombyx mori* [36, 42]) probably does not contain other upstream promoter elements, such as Sp1 or ATF, because it would then resemble a class II promoter and have the potential to be transcribed by RNA polymerase II. We suggest that the EBER 2 ETAB promoter element has evolved not only to exploit the ability of the TATA box and whatever interacts with it to induce transcription with the aid of proteins binding to other upstream promoter elements but

also to permit the promoter to be transcribed by only RNA polymerase III. This is important for the expression of the EBER genes for several reasons. First, the transcription of class III genes is usually not individually regulated; for example, the protein E1A activates transcription of all class III genes by modifying the general RNA polymerase III transcription factor TFIIC (24). Conversely, the additional upstream promoter elements of the EBER genes probably permit them to be regulated individually; after EBV infection of B lymphocytes, the EBERs are transcribed later than the other latently expressed viral genes (53). Second, the EBERs along with other class III gene products are short RNAs whose defined length is achieved with RNA polymerase III because it has a specific termination signal.

What binds to the ETAB promoter element? Among known proteins that might interact with the ETAB promoter element, the two obvious candidates are TBP and TFIIB. TBP, which functions in conjunction with the TATA box sequence, is necessary for the first step in the initiation of RNA polymerase II transcription (reference 10 and references therein). There is precedent for TBP functioning in RNA polymerase III gene expression. The yeast U6 promoter requires TBP (40); however, the need for a TATA box in the yeast U6 promoter is not conclusive (8). Recently, transcription of the vertebrate U6 promoter, which contains an essential TATA box, has been found to require TBP (39, 57). Although the ETAB promoter element cannot substitute for a class II TATA box and may actually prevent RNA polymerase II transcription, EBER 2 transcription probably requires TBP. TBP could bind directly to the ETAB promoter element and then bind TBP-associated factors specific for RNA polymerase III transcription. Alternately, another upstream promoter element-binding protein, such as Sp1, could facilitate the binding of TBP to the ETAB promoter element in a manner similar to that of the class I rRNA promoter and other genes lacking a TATA box (14, 50).

The other possibility is that TFIIB binds directly to ETAB (4). The binding of TFIIB at -10 to -40, centered around the location of a possible TATA box (31, 32), requires TFIIC to first bind over boxes A and B. Since boxes A and B are present and functional in the EBER 2 gene, the ETAB promoter element, which is located in a region where a normal tRNA gene binds TFIIB, may also interact with this protein complex. However, it is unclear why a specific ETAB sequence would be required, since a consensus sequence in this region in other class III genes has not been found.

The 7SL gene has an ETAB promoter sequence. The EBER and HVP1 and HVP2 small RNA genes are probably not the only class III genes which have an ETAB promoter element. The cellular gene 7SL, which contains a functional upstream ATF promoter element (6, 7), also has a TATA box sequence. The 7SL TATA box is similar in location as well as in sequence requirements to the ETAB promoter element; it contains G residues at positions 3 and 6 and a T doublet at the same positions as the ETAB element (Fig. 4). The 7SL TATA box undoubtedly functions in 7SL transcription like the ETAB promoter element in EBER 2 gene transcription, but this has not been shown experimentally. An examination of the upstream sequences of other class III genes which have been found to require upstream sequences for their transcription (references in reference 21; 39) and TATA box sequences from a compilation of 168 eucaryotic RNA polymerase II promoters (9) discovered no other sequences with ETAB promoter element characteristics.

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FIG. 4. Sequence comparison of the upstream regions of EBER 1 and 2, HVP1 and HVP2, and 7SL (58)

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