Efficient transcription of the EBER2 gene depends on the structural integrity of the RNA

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ABSTRACT

A 3'-truncated EBER2 RNA gene, although containing all previously identified promoter elements, revealed drastically reduced transcription rates *in vitro* and *in vivo* when fused to a heterologous terminator sequence. Inactivations were also observed with double point mutations affecting 5'- or 3'-end sequences of the EBER2 gene. However, wild-type activity of these mutants could be restored by compensatory mutations of the opposite strand of the EBER2 RNA sequence. A similar rescue was achieved with the 3'-truncated EBER2 gene, if the heterologous terminator was adapted for complementarity to the initiator element of the construct. Yet, double-strandedness alone of the RNA ends was not sufficient for high transcriptional activity of these gene constructs. Rather, the use of a nonrefoldable spacer, separating the 5'- and 3'-stem-loop structures, demonstrated that spatial proximity of the ends of EBER2 RNA was required. Furthermore, decay kinetics of wild-type and mutant RNA synthesized *in vitro* indicated that the effects observed could not be explained by altered transcript stability. Finally, single-round transcription confirmed that the reduced expression of mutant genes was not caused by decreased primary initiation reactions. In addition, differential sarcosyl concentrations demonstrated that the rate of reinitiation clearly was affected with the mutant EBER2 genes. Together, these results indicate that the secondary structure of this viral RNA represents a major determinant for efficient transcription of the EBER2 gene by host cell RNA polymerase III.

Keywords: Pol III transcription; EBER2 RNA; RNA secondary structure; transcriptional regulation

INTRODUCTION

Latent infection of human B lymphocytes by Epstein-Barr virus (EBV) is accompanied by effective transcription of two viral genes coding for small nonpolyadenylated RNAs called EBER1 and EBER2 (for review, see Clemens 1994). These genes are transcribed by RNA polymerase III (Howe and Shu 1989, 1993), and the transcripts accumulate to high levels (up to 10^7 copies) within the infected cells (Lerner et al. 1981). The precise function of these small viral RNAs remains to be elucidated (Teramoto et al. 1998). However, the analogy with the adenovirus-associated VAI and VAI RNAs (Rosa et al. 1981; Sharp et al. 1993) and the localization of the EBERs (Schwemmle et al. 1992) point to a role for these small RNAs in translational control. Furthermore, during the lytic phase of viral replication, transcription of the EBER1 and EBER2 genes is down-regulated (Greifenegger et al. 1998), and the transcripts appear to reveal signifi-

cantly prolonged half lives, as compared with those during latent infection (Clarke et al. 1992).

Transcription by RNA polymerase III of several eukaryotic genes (Pol III genes) has been studied in detail with respect to promoter structures as well as the involvement of general and gene-specific transcription factors (for review, see Geiduschek and Tocchini-Valentini 1988; White 1994). Generally, promoters of Pol III genes reveal a remarkable heterogeneity. Therefore, according to their promoter structure these genes have been classified into four groups (Willis 1993). Together with the mammalian 7S L (srp) RNA genes, the Epstein-Barr-virus-encoded EBER1 and EBER2 genes form a group that is characterized by a split promoter, localized inside as well as upstream of the transcribed sequence. From *in vivo* and *in vitro* transcription analyses, it appears that the structures of human 7S L RNA and the two EBER RNA gene promoters are largely identical (Ullu and Weiner 1985; Howe and Shu 1989, 1993; Bredow et al. 1990a,b; Müller and Benecke 1999).

In addition to its previously recognized role as a repository of genetic information and as an essential carrier of this information within the cell, RNA displays an extreme functional versatility that is based on diverse and highly specific

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interactions with other macromolecules. Among the large variety of such interactions, the involvement of RNA structure in transcriptional regulation has been studied in a number of prokaryotic as well as eukaryotic systems (for review, see Platt 1998). In particular, the transcriptional activation of HIV mRNA synthesis by interaction of the TAR element with the virally encoded Tat protein (for review, see Jones and Peterlin 1994) has attracted considerable interest.

In this report, we describe another example of transcriptional activation of a viral gene by the structure of the newly synthesized RNA. In contrast to the elongation control mediated by the TAR element located near the 5'-end of HIV mRNA, efficient transcription of the EBER2 gene is regulated by the folded structure of the completed transcript and seems to depend on the spatial proximity of the 5'- and 3'-ends of the RNA. Finally, we provide compelling evi-

dence that reinitiation events are involved in the control of the EBER2 transcription.

RESULTS

Transcription in vitro and in vivo of chimeric EBER2 gene constructs

To examine transcription initiation on the EBER2 promoter in more detail, a chimeric gene was cloned that contained the entire EBER2 promoter region (−159 to +80) fused to a heterologous Pol III termination signal. This terminator sequence consisted of the 3'-terminal stem-loop structure of the human 7S K RNA (+270 to +331) studied in our laboratory previously (Kleinert and Benecke 1988). The resulting construct, 3'K (Fig. 1A), was analyzed for transcriptional activity, in comparison with the EBER2 wild-type (wt) gene. Analyses were performed in duplicate throughout. In vitro transcription reactions in HeLa cell nuclear extracts (Fig. 1B) gave rise to specific transcripts of the expected length of 172 nt (EBER2) and 144 nt (3'K), respectively. In both cases, synthesis of these labeled RNA molecules was sensitive to high concentrations of α -amanitin (200 μ g/mL), indicating that they represented RNA polymerase III-specific transcripts (data not shown). However, a quantification of the radioactivity associated with the individual bands revealed a significantly reduced activity of the 3'K fusion construct (42%; Fig. 1B, lanes 3,4), as compared with the wild-type gene (100%; Fig. 1B, lanes 1,2). Furthermore, a much more pronounced inactivation was observed in vivo, after transient transfection of cultured cells. Under these conditions the chimeric gene was almost totally inactivated (Fig. 1C). Normalization for the activity of a cotransfected reference gene (Fig. 1C, ref) of the signals obtained with the EBER2 wild-type gene (Fig. 1C, lanes 1,2) and the chimeric gene (Fig. 1C, lanes 3,4) confirmed that in vivo expression of the 3'K fusion construct had dropped to a residual activity of ~5%.

Analysis of mutant genes affecting the secondary structure of the EBER2 transcripts

The results described above for the EBER2/7S K RNA fusion construct

FIGURE 1. Transcription in vitro and in vivo of a chimeric EBER2/7S K RNA (3'K) gene construct. (A) Schematic presentation of the fusion gene. (B) Duplicate analyses of in vitro transcription in HeLa cell nuclear extracts of the EBER2 wild-type gene (lanes 1,2) and the 3'K construct (lanes 3,4). Analysis of labeled transcripts was in 6% polyacrylamide gels containing 8 M urea. (C) Same as B; in this case, however, transcription analysis was in vivo, after transient transfection of HepG2 cells. Analysis of transcripts was performed by S1-nuclease protection of labeled template DNA fragments (arrow). For normalization, a reference plasmid (see Materials and Methods) was cotransfected and analyzed in parallel (ref). (m) Labeled marker DNA fragments.

(3'K) were quite unexpected, because that chimeric gene included the entire promoter region of EBER2. Therefore, the question arose whether the structure of the resulting transcripts might have caused the loss of transcriptional activity observed with the fusion construct. That hypothesis was examined with mild structural variants of the EBER2 wild-type RNA. For this, double point mutations were introduced into the transcribed sequence of the EBER2 gene, yet clearly outside the known internal promoter elements, that is, the A- and B-boxes located between positions +12/+22 and +50/+60, respectively (Howe and Shu 1989). In the first mutant (+6,9), the A and C nucleotides located at positions +6 and +9 of the wild-type sequence were replaced by C and G residues, respectively. As is evident from the partial sequence shown in Figure 2A, these two mutations affect the double-stranded stem structure formed by base pairing of the 5'- and 3'-termini of EBER2 RNA. The *in vitro* transcription analysis of this mutant gene is shown in Figure 2B. In comparison with the wild-type (Fig. 2B, lanes 1,2), the +6,9 mutant gene (Fig. 2B, lanes 3,4) again revealed a significantly reduced template activity of ~33%. (The reference band [Fig. 2B, ref] taken here for normalization represents endogenous U6 snRNA, labeled by a U6-specific terminal uridylyl transferase activity; Trippe et al. 1998.) Furthermore, transient transfection of cultured cells confirmed the reduced transcriptional activity of the +6,9 mutant *in vivo* (Fig. 2C, cf. lanes 1,2 and lanes 3,4). In contrast to the activity observed with the fusion construct before (Fig. 1C), *in vivo* transcription of the +6,9 mutant

(23% of wild type) was clearly above basal level. It is quite conceivable that this higher residual activity reflects the less severe structural alteration of this mutant gene.

To confirm our assumption that a structural variation caused the reduced transcriptional capacity observed before, additional mutations were introduced into the 3'-terminal sequence of the EBER2 RNA. These mutations aimed at the restoration of the 5'-3' stem structure observed in EBER2 wild-type RNA. For this, the nucleotide exchanges of the +6,9 mutant were compensated by C and G residues at positions +156 and +160, respectively. These compensatory mutations (construct +6,9/+156,160) indeed restored wild-type levels (102% and 99%, respectively) of transcriptional activity, when analyzed *in vitro* and *in vivo* (see lanes 5,6 of Figs. 2B and 2C, respectively). Finally, these compensatory mutations alone (+156,160) were tested in the context of the EBER2 gene. Albeit clearly above the +6,9 values, the reduced activities observed here *in vitro* and *in vivo* (53% and 42% of wild type, respectively; see lanes 7,8 of Figs. 2B and 2C) confirmed that either of the two one-sided mutations of the EBER2 RNA alone mediated a deleterious effect on transcription, which, however, was definitely suppressed by restoring RNA sequence complementarity. The slightly stronger inhibition of the +6,9 mutant, compared with the +156,160 mutant, might be due to an interference of the former mutation with a putative Pol III initiator element identified at the transcription start site of the human 7S K RNA gene (Sandrock and Benecke 1999).

FIGURE 2. Transcriptional activity of EBER2 wild-type and mutant genes. (A) Partial secondary structure of EBER2 +6,9 mutant gene. In this mutant, the +6-nt and +9-nt (A and C) were replaced by C and G (bold) residues, respectively. (B) Duplicate *in vitro* transcriptions of the EBER2 +6,9 mutant gene (lanes 3,4) in comparison to the wild-type (wt) gene (lanes 1,2). The EBER2 +6,9/+156,160 mutant gene (lanes 5,6) contains two additional 3'-end opposite-strand mutations of the RNA, compensating for the original +6,9 mutations at the 5'-end. The +156,160 mutant (lanes 7,8) shows these 3' compensatory mutations alone, in the context of the 5'-terminal EBER2 RNA wild-type sequence. Labeled transcripts (arrow) were analyzed as in Figure 1B. (ref) Nuclear U6 snRNA, labeled by a U6-specific terminal-uridylyl-transferase, present in HeLa cell nuclear extracts (Trippe et al. 1998). (C) Same as B; however, in this case, expression of genes was studied *in vivo*, after transient transfection of HepG2 cells and transcript analysis by S1-nuclease protection. (ref) The signals obtained with RNA transcribed from a cotransfected reference template, required for normalization.

Because structural variants may affect RNA stability, particularly if double-stranded ends are involved, decay kinetics of in vitro synthesized RNA were analyzed. For this, transcription reactions were terminated by the addition of α -amanitin (200 μ g/mL), and the assay was further incubated for the times indicated in Figure 3. The degradation curves were obtained by PhosphorImager quantification of the signals shown in the inserts for wild-type and mutant (+6,9) EBER2 RNA. Although the overall turnover was rather low, the data observed fit well to an exponential function (solid line), as would be expected for first-order decay kinetics. These results confirmed that in this in vitro system, ribonuclease activity was not a major factor. Furthermore, the run of both curves was almost in parallel, indicating that approximately the same amount of labeled RNA was degraded. Finally, assuming that for both RNAs the rate of synthesis had been identical, the decay observed with the mutant RNA molecules could not account for the strongly reduced signals observed for +6,9 at zero time. Therefore, reduced expression of +6,9 mutant RNA cannot be attributed to altered decay rates.

Rescue of EBER2/7S K RNA fusion constructs by introducing sequence complementarity

To see whether complementarity per se, that is, independently of the authentic EBER2 sequence, is required for efficient expression of the gene, rescue experiments were performed with the 3'K fusion construct studied before. A close examination of 5'-EBER2 (+1 to +7) and 3'-7S K (+320 to +325) RNA sequences revealed that insertion of a U nucleotide at position +323 of 7S K RNA would generate a 7-bp sequence complementarity between the two ele-

ments. The last 36 nt of the human 7S K RNA can form a distinct stem-loop structure (see Fig. 4A). Because that sequence element was found highly conserved during evolution (Gürsoy et al. 2000), it appears that this stem-loop structure does really exist. Therefore, to facilitate base pairing between the (5') EBER2 and the (3') 7S K RNA sequence, that 7S K-hairpin structure had to be disrupted. This was achieved by three noncomplementary residues at positions +303, +304, and +310, respectively. Together with the U-nucleotide insertion at position +323, the resulting construct allowed stable duplex formation between the 5'-EBER and 3'-7S K RNA sequences (Fig. 4A, 3'K + U).

A second rescue mutant of the EBER2/7S K fusion construct was generated by adaptation of 7 nt immediately upstream of the 3'-7S K hairpin structure to the first 7 nt of EBER2 RNA. Now, both ends of the RNA transcribed from this fusion construct (3'K_{mut}; Fig. 4A) should be engaged again in double strands. In contrast to EBER2 wild-type RNA and to the former rescue construct, however, the 5'- and 3'-ends of this 3'K_{mut} RNA do not belong to the same stem structure. It should be noted that these two rescue constructs, like the basal 3'K fusion construct, retained the authentic 5'-EBER2 sequence (5'-wt). Therefore, we also wanted to analyze the effect of the previously described +6,9 EBER2 mutations in the context of these mutated 3'K sequence elements. The corresponding +6,9 mutants of those three EBER2/7S K fusion constructs were easily accessible, by cloning via the *Sma*I restriction site at position +77 of the EBER2 sequence.

The transcriptional activity of these six fusion constructs was analyzed in comparison with the EBER2 wild-type and the +6,9 mutant genes. In vitro transcription results are summarized in Figure 4B. As before, the basic 3'K fusion construct (Fig. 4B, lanes 5,6) was clearly less active (here 28%) than the EBER2 wild-type gene (Fig. 4B, lanes 1,2). In contrast, insertion of the U nucleotide and disruption of the 7S K 3'-stem structure resulted in significant recovery of the basic 3'K fusion construct. Transcriptional activity of this 3'K + U construct was restored to the level of the EBER2 wild-type gene (Fig. 4B, cf. lanes 9,10 with lanes 1,2). Furthermore, the 3'K_{mut} construct (Fig. 4B, lanes 11,12) was also found clearly above the level of the basic 3'K clone. Yet, with an apparent activity of 49%, this second rescue construct clearly fell short of the EBER2 wild-type gene. A different pattern was observed with the additional +6,9 EBER2 mutations. In the context of the basic 3'K fusion construct, these additional mutations had no further deleterious effect (Fig. 4B, cf. lanes 7,8 with

FIGURE 3. Decay of in vitro transcribed wild-type (wt) or +6,9 mutant EBER2 RNAs. In vitro transcription in nuclear extracts was at 30°C for 30 min. At this point (0 time), 200 μ g/mL α -amanitin was added, and the assay was further incubated at 30°C for the times indicated. Aliquots were taken at 5-min intervals and processed for electrophoretic analysis, as before. Individual points of the curves were obtained after PhosphorImager quantification of the bands shown in the inserts, respectively. (PSL) Photo-stimulated luminescence.

lanes 5,6). It is obvious that now the inhibitory effect of the +6,9 point mutations was eliminated. In contrast, both rescue mutants, 3'K + U (Fig. 4B, lanes 13,14), as well as 3'K_{mut} (Fig. 4B, lanes 15,16), were clearly sensitive again to the introduction of the additional +6,9 EBER2 mutations at their 5'-ends.

The analysis in vivo of those mutants was in full agreement with the in vitro data, yet revealed much more pronounced effects again. For an easier interpretation, the signals of Figure 4C were analyzed by PhosphorImager quantification and normalized for the references (ref). As is evident from the diagram in Figure 4D, these in vivo results confirm two major findings: the first being that in comparison with the basic 3'K fusion construct, both rescue

clones (3'K + U and 3'K_{mut}) revealed significantly increased activities (Fig. 4B, cf. lanes 9,10 and 11,12 with Fig. 4C, lanes 5,6). In this case, however, both constructs did not reach the level of the EBER2 wild-type gene (Fig. 4C, lanes 1,2). Again, the increase in template activity observed with the 3'K + U construct was almost twice that of 3'K_{mut} (Fig. 4C, lanes 9–12). Secondly, the additional +6,9 mutations of the EBER2 5'-sequence were only found deleterious for the two rescue constructs, yet not for the basic 3'K fusion clone. Together, these results confirm our previous conclusion that the negative effect observed with different EBER2 mutant constructs was caused by an interference with formation of double-stranded terminal regions within the newly transcribed RNA.

FIGURE 4. (Continued on next page)

The spatial vicinity of the 5'- and 3'-ends of EBER2 RNA is required for efficient transcription

The observation that the 3'K_{mut} rescue construct reproducibly was found less active than the 3'K + U template indicated that the formation of double-stranded ends alone might not be sufficient. It rather appeared that the relative structural arrangement toward each other of 5'- and 3'-ends of the RNA also might be crucial for efficient transcription. Therefore, a structural variant of the 3'K_{mut} construct was generated by insertion of a nonrefoldable CA dinucleotide sequence element (see Fig. 4A). Two different constructs were analyzed, with 10, (CA)₅, or 20, (CA)₁₀, extra nucleotides separating the two terminal stem structures. The analysis in vivo of these two clones, in comparison with the 3'K_{mut} (upper level) and 3'K (lower level) fusion constructs, is shown in Figure 5A. The activity of these two constructs in vivo (Fig. 5A, lanes 7–10) revealed that insertion of these CA spacer elements clearly eliminated the rescue effect of the 3'K_{mut} clone (Fig. 5A, lanes

5,6) and reduced the activity down to that of the basic 3'K fusion construct (Fig. 5A, lanes 3,4). In agreement with the basic 3'K fusion construct, again the low activity of the (CA)₅ and (CA)₁₀ templates was not further reduced by the additional +6,9 mutations of the EBER2 5'-sequence. As a control, the effect of the (CA)₅ spacer element was tested in the context of the EBER2 wild-type and +6,9 mutant genes (inserted at +77 of the EBER2 RNA sequence). As is shown in Figure 5B, the activity of the EBER2 wild-type gene was also reduced by the insertion of that spacer (Fig. 5B, lanes 5,6). Yet, with 66% of wild-type activity, this inhibition was significantly less severe than that observed before, in the context of the 3'K_{mut} clone. Once again, the additional +6,9 mutation (Fig. 5B, lanes 7,8) virtually abolished transcription from that EBER2 mutant gene. Together, these results indicate that not just the double-strand engagement of the 5'- and 3'-ends of EBER2 RNA is important, but, rather, a close spatial vicinity of both ends is required for efficient transcription of the EBER2 gene.

FIGURE 4. Analysis of EBER2/7S K RNA rescue constructs. (A) Schematic presentation of putative secondary structure of 3'-terminal sequences. 3'K is the authentic 3'-end of the human 7S K RNA. 3'K + U is the basic EBER2/7S K RNA fusion construct, however, with insertion of a U residue at position +323 of the 7S K sequence, generating a 7-bp complementarity between the 5'-end of EBER2 RNA and the 3'-end of 7S K RNA. 3'K_{mut} is also an EBER2/7S K RNA fusion construct; in this case, however, the 7 nt immediately preceding the 3'-terminal stem-loop structure of 7S K RNA were adjusted to sequence complementarity with the 5'-terminal EBER2 RNA. The arrow with (CA)_{5/10} indicates the position where a nonrefoldable spacer was inserted (see Fig. 5). (B) In vitro transcription analysis of the rescue constructs 3'K + U (lanes 9,10,13,14) and 3'K_{mut} (lanes 11,12,15,16) in comparison with the basic EBER2/7S K RNA fusion construct (3'K, lanes 5–8). Each of the fusion constructs was analyzed in the context of the 5'-terminal EBER2 wild-type (wt) sequence (lanes 5,6,9–12) or the +6,9 mutant sequence (lanes 7,8,13–16). For a comparison, lanes 1–4 show the activities of the EBER2 RNA wild-type (lanes 1,2) or +6,9 mutant (lanes 3,4) genes. (C) Same templates as before. In this case, however, transcription analysis of constructs was in vivo, after transient transfection of HepG2 cells and analysis of transcripts by S1-nuclease protection (see arrow). (ref) As in Figures 1 and 2C, represents protected bands of reference transcripts required for normalization. (D) Schematic presentation of the results in parts B and C, after PhosphorImager quantification of the bands observed, as described. Error bars as obtained from the duplicates are indicated.

FIGURE 5. (A) Expression in vivo of chimeric EBER2/7S K fusion constructs with a spacer separating the 5'- and 3'-ends of the resulting transcripts. The 3'K_{mut} (see Fig. 4A) rescue template was further modified by introduction of 5 or 10 CA dinucleotides, as indicated by the arrow, generating the 3'K_{mut}(CA)₅ or 3'K_{mut}(CA)₁₀ constructs, respectively. These constructs (lanes 7–10) were analyzed in comparison with the basic 3'K fusion construct (lanes 3,4) or the 3'K_{mut} clone (lanes 5,6). In each case, the 5'-terminal section of the constructs either consisted of the EBER2 RNA wild-type (wt; lanes 1,3,5,7,9) or the +6,9 mutant sequence (lanes 2,4,6,8,10). Again, EBER2 RNA-specific bands protected against S1-nuclease digestion are indicated by an arrow; (ref) reference transcripts from the cotransfected template. (B) Expression in vivo of the EBER2 gene and the +6,9 mutant, both with a 5-CA-dinucleotide sequence element inserted at position +77 [wt/(CA)₅, lanes 5,6; +6,9/(CA)₅, lanes 7,8]. Constructs were analyzed in comparison with the EBER2 wild-type gene (lanes 1,2) and the +6,9 mutant (lanes 3,4). Again, S1-nuclease-digestion-protected EBER2 RNA-specific bands are indicated by an arrow; (ref) reference transcripts.

Multiple and single-round transcription of wild-type and mutant EBER2 RNA genes

To address the question by which mechanism the structure of the RNA contributes to the rate of transcription, we analyzed initiation as well as reinitiation events. For this, differentially inhibitory concentrations of sarcosyl were applied. The initial experiments performed with the EBER2 wild-type gene (Fig. 6) essentially confirmed earlier results obtained for Pol III transcription of the adenoviral VA I RNA gene (Kovelman and Roeder 1990). Recruitment of transcription complexes on EBER2 templates was already affected by sarcosyl concentrations of 0.005%, and no initiation took place any more at inhibitor concentrations exceeding 0.01% (Fig. 6A, upper panel). In contrast, if sarcosyl was added only after preinitiation complex formation, inhibition of reinitiation required clearly higher concentrations and started at 0.015% (Fig. 6A, lower panel). The time course of full-length EBER2 transcription (0.08% sarcosyl added after preincubation) is shown in Figure 6B. This single-round transcription experiment revealed that, in the absence of reinitiation, synthesis of EBER2 RNA was completed after 1 min. Based on this general setup, the in vitro transcription of EBER2 mutants was studied in comparison

with the wild-type gene. As is evident from Figure 7A, the reduced capacity for transcription under control conditions of the +6,9 mutant (Fig. 7A, lanes 3,4), in comparison with the wild-type gene or the +6,9/156,160 rescue construct (Fig. 7A, lanes 1,2 and 5,6), is also retained if only reinitiation is allowed (0.015% sarcosyl) after one first round of initiation (Fig. 7A, lanes 7–12). In agreement with these results, the formation of the primary initiation complex on mutant gene constructs was not altered. This became evident from the single-round transcription analyses with wild-type and mutant genes in Figure 7B. Essentially all mutant constructs (Fig. 7B, lanes 3–10) revealed the same template activity as the EBER2 wild-type gene (Fig. 7B, lanes 1,2). Only a minor reduction was observed for the +6,9 mutant (Fig. 7B, lanes 3,4), which is explained by fact that the +6,9 mutation also affects the initiator element, as discussed above. That conclusion is supported by the intriguing finding that the 3'K construct, found to be the least active template throughout this study (cf. Figs. 1 and 4), virtually revealed no reduced capacity for single-round transcription (Fig. 7B, lanes 7,8). Together, the sarcosyl experiments allow two major conclusions: first, the reduced transcription rates of the mutant EBER2 genes are not caused by decreased template activity; and, second, the

FIGURE 6. (A) Transcription in vitro of the EBER2 wild-type gene in the presence of sarcosyl. Increasing concentrations of sarcosyl as indicated were either present during a 30-min preincubation period (upper panel) or added after that time (lower panel). (B) Single-round transcription of the EBER2 wild-type gene with 0.08% sarcosyl being added after preincubation to prevent any further initiation of transcription, including reinitiation. In this case, autoradiography was for 6 d.

structural mutation of the EBER2 RNAs significantly impairs the reinitiation reaction of Pol III transcription on the same template.

DISCUSSION

The results described here establish a structural requirement of the RNA product for efficient transcription of the EBER2 gene in vitro and in vivo. Up to now, only very few examples have been found for the involvement of newly synthesized RNA in transcriptional regulation of its own gene. So far, the best studied system is the Tat-responsive (TAR) element of HIV-1 messenger RNA and its role in mRNA biogenesis (Jones and Peterlin 1994). In that case, regulation is mediated by an RNP complex consisting of the TAR element located near the 5'-end of the mRNA, the viral Tat protein, and the pTEFb (CDK9/cyclin T; Wei et al. 1998). The regulation by TAR of HIV mRNA synthesis is achieved via elongation control of the transcribing RNA polymerase II. Furthermore, that regulation clearly depends on a strict requirement also for the native primary structure around the bulged core of the TAR element.

A second example of transcriptional regulation by the nascent RNA chain has been described for the human 7S L (srp) RNA gene (Emde et al. 1997). There, a complex pro-

pellier-like secondary structure at the very 5'-end of 7S L RNA is required for efficient transcription by RNA polymerase III. That structure also includes a tetranucleotide bulge and has been shown to be recognized by cellular proteins. As in the case of EBER2 RNA, mutations disrupting the double strands of that 7S L RNA structure resulted in pronounced reduction of transcription in vivo and in vitro and could be compensated by opposite-strand mutations of the RNA sequence. As here, the extent of inactivation and rescue could not be explained by altered transcript stabilities (Emde et al. 1997), although it cannot totally be excluded that in both cases some minor degradation effect also might contribute to lowered expression rates.

The most intriguing question was by which mechanism the structure of the RNA product could be involved in control of transcription efficiency. In analogy with the viral Tat-TAR interaction, the described Pol III systems too might result in control of elongation. However, experiments with RNA polymerase III molecules synchronized on wild-type and mutant 7S L RNA genes revealed no differential elongation rates of Pol III transcription with these templates (G. Emde and B.J. Benecke, unpubl.). Furthermore, the sarcosyl experiments described here provide direct evidence that efficient reinitiation by Pol III depends on appropriate RNA structures. In this context, it is interesting

FIGURE 7. (A) Transcription in vitro of the EBER2 wild-type (wt) gene (lanes 1,2), the +6,9 mutant (lanes 3,4), and the compensatory +6,9/+156,160 mutant (lanes 5,6). The same reactions were performed with 0.015% sarcosyl added after the preincubation period to prevent primary transcription initiation: EBER2 wild-type gene (lanes 7,8), +6,9 mutant (lanes 9,10), and +6,9/+156,160 mutant (lanes 11,12). (B) Single-round transcription performed in duplicate with EBER2 wild-type (lanes 1,2), +6,9 mutant (lanes 3,4), and +6,9/+156,160 mutant (lanes 5,6) genes or the 3'K (lanes 7,8) and 3'K+U (lanes 9,10) constructs.

to note that facilitated recycling pathways for RNA polymerase III have been described in different systems (Gottlieb and Steitz 1989; Maraia et al. 1994; Dieci and Sentenac 1996). Those results were attributed to a termination-dependent commitment for the same Pol III template and, in some cases, were attributed to the action of the La protein (Gottlieb and Steitz 1989; Maraia et al. 1994). In our inactivated mutant constructs, however, the oligo-uridylic acid terminator sequences were not affected, as evidenced for example by the +6,9 and 3'K_{mut} constructs.

At first glance, the structural arrangements of 5'- and 3'-termini of EBER2 and 7S L RNA appear to be quite different. In case of the viral RNA, both ends belong to the same stem structure, whereas in the latter case, 5'- and

3'-ends are engaged in different double-stranded domains. Yet, within the chimeric EBER2/3'K construct, adaptation of the 3'K sequence immediately preceding the terminal stem-loop structure for complementarity to the 5'-EBER2 sequence resulted in a secondary structure of termini that is reminiscent of 7S L RNA. That construct (3'K_{mut}) revealed significant, albeit not full rescue in template activity. In view of the identity in promoter structure of EBER2 and 7S L RNA genes, it is tempting to speculate that the large difference in transcriptional efficiency observed between both genes *in vivo* and *in vitro* (E. Dümpelmann, unpubl.) may be attributed to this divergent structural arrangement of the respective RNA termini. This conclusion is in agreement with the stronger rescue effect of the chimeric EBER2/3'K

construct by adaptation of the 5'-EBER sequence to the very 3'K sequence (3'K + U), as compared with that of the 3'K_{mut} construct. Finally, the significance for transcriptional efficiency of the spatial arrangements of the 5'- and 3'-ends of transcripts is unambiguously confirmed by the full inactivation observed upon introducing the nonrefoldable oligomeric (CA) elements (see Fig. 5).

In this context, it is interesting to note that long ago Doty and coworkers had already pointed out that under physiological conditions, complementary sequences of single-stranded RNA molecules will form double-helical structures to something like the maximum extent possible (Doty et al. 1959). That theory is supported by the calculated secondary structure of EBER2 RNA, as shown in the upper panel of Figure 8. That secondary structure, as calculated by the Mfold program (Zuker 1989), differs in various parts from those published earlier (Rosa et al. 1981; Glickman et al. 1988). However, all structures agree on placing the 5'- and 3'-ends of EBER2 RNA into a common stem structure. Interestingly, the chimeric 3'K + U construct, although composed of two unrelated sequence elements, also can form a secondary structure closely related to that of EBER2, with both RNA termini again arranged immediately adjacent to each other (Fig. 8, bottom panel). Any such thoroughly folded RNA structure, as generally observed with small stable RNA molecules, will necessarily bring the two ends into close spatial vicinity. At least in the Pol III

FIGURE 8. Potential secondary structures of EBER2 wild-type (*upper*) and the chimeric 3'K + U construct (*lower*) RNA sequences as calculated by the Mfold program (Zuker 1989).

transcription system, it appears that this arrangement might provide the molecular basis for a facilitated reinitiation process. A hypothetical model might consist in a relocation of the terminated RNA polymerase III to the initiation site by spontaneous folding of the newly transcribed RNA. Finally, we conclude that the structural involvement of nascent RNA in transcriptional regulation described for human 7S L RNA previously (Emde et al. 1997) and for EBER2 here may reflect a more general phenomenon of the eukaryotic Pol III transcription system.

MATERIALS AND METHODS

Templates

The EBER2 RNA gene was cloned by PCR amplification from the Epstein-Barr Virus *cM SalA* fragment, kindly provided by G. Laux (Munich). The gene contained the entire 172 bp of transcribed sequence plus 160 bp of 5'-upstream and 28 bp of 3'-flanking sequences, respectively. The basic EBER2/3'K fusion construct contained the EBER2 RNA fused to the *SmaI* restriction site at +80, with the 3'-terminal human 7S K RNA gene sequence (+270 to +331) being fused to that site via 3 bp of polylinker sequence. Mutations were introduced by replacement of the respective sequences with synthetic oligonucleotides.

In vitro transcriptions

HeLa nuclear extracts (7 mg/mL protein) were prepared according to Dignam et al. (1983). In vitro transcription reactions with 10 μ L of extract, 1 μ g of template, and 5 μ Ci of [α - 32 P]UTP (800 Ci/mmol; New England Nuclear) as well as extraction of RNA, gel electrophoresis, and autoradiography have been described in detail previously (Emde et al. 1997). The kinetics of decay were monitored after in vitro transcription for 30 min (0 time), addition of an excess of α -amanitin (200 μ g/mL; Roche), and further incubation at 30°C for the times indicated. Single-round transcription and analyses with differential sarcosyl concentrations were performed according to Kovelman and Roeder (1990).

Transfections and S1-nuclease protection analysis

Hepatoma HepG2 cells were transfected for 36 h with 5 μ g of plasmid DNA via calcium-phosphate coprecipitation. For reasons of normalization, a reference plasmid (20 μ g) consisting of the human 7S K RNA gene promoter and a vector sequence as reporter (K+8/pAT; Sandroock and Benecke 1999) was cotransfected. Extraction of cellular RNA, hybridization to labeled probes, S1-nuclease digestion, and analysis of protected fragments were performed as described by Emde et al. (1997). Reference transcripts were detected with a 3'-end-labeled probe (*Clai* site) and gave rise to a protected band of 215 nt. To be able also to detect EBER2 transcripts containing the +6,9 mutation, a special probe was cloned that was homologous to EBER2 RNA only between +10 and the *SmaI* site (+77), which in turn was used for 5'-end-labeling. That probe gave rise to a protected fragment of 67 nt in length. Autoradiography was performed for 16 h with Kodak X-

ray films, using Cronex intensifier screens. The relative activities described in the text and as presented in Figures 3 and 4D were obtained by PhosphorImager quantification (Fuji BAS 1000; TINA 2.09) of the individual bands detected in dried gels, with background subtraction throughout.

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