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In vitro transcription of two Epstein-Barr virus specified small RNA molecules

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ABSTRACT

Cloned DNA from the EcoRI J fragment of EBV has been used as template for in vitro transcription experiments using cell-free extracts prepared from HeLa or KB cells. Two EBV specific RNAs each about 175 bases in length were synthesised and nuclease S1 mapping experiments determined that these in vitro products corresponded precisely to the in vivo species obtained from Raji cells. These two RNA molecules are transcribed by RNA polymerase III and in common with other pol III-synthesised RNAs the coding sequences contain intragenic control regions. The relative abundance of the two RNAs synthesised in vitro differs from that observed in vivo.

INTRODUCTION

In recent years investigations into the transcription patterns of Epstein-Barr Virus (EBV) related RNA in EBV transformed cells have been undertaken in several laboratories (1-5). It was first shown by Rymo (1) that the most abundant EBV-related cytoplasmic RNA in such cells was complementary to a small region of the EBV genome defined by the EcoRI J fragment. This result was subsequently confirmed in other laboratories (4,5,6) and finer mapping studies defined the major transcriptionally active region of EBV DNA as lying within a stretch of about 700 base pairs close to the right end of EcoRI J (5) on the standard physical map (7). A further, more surprising finding was that this major RNA consisted of two distinct, non polyadenylated species about 170 bases in length (5,8).

In this report we show that EBV EcoRI J DNA cloned in the plasmid pBR322 can be transcribed in vitro to give two RNAs which initiate and terminate in precisely the same locations of the EBV genome as the major in vivo species isolated from Raji cells. However in vitro synthesis of one of the RNAs vastly exceeds that of the other. In common with small RNA molecules obtained from several other sources, the EBV derived species are transcribed using RNA polymerase III and their coding sequences appear to contain intragenic promoter and/or control regions. Whilst this work was

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in progress a report appeared (8) which also demonstrated in vitro transcription of the two small EBV specified RNAs.

MATERIALS AND METHODS

a) Lymphoid cells, preparation and labelling of cytoplasmic RNA

Cells from the Burkitt's lymphoma-derived line, Raji (9), were grown in RPMI 1640 medium supplemented with 5% foetal bovine serum (Gibco). Total cytoplasmic RNA was prepared using standard procedures (10) and where necessary was labelled in vitro with ^{32}P as previously described (5).

b) Plasmid DNA

Plasmids pBR322 (11), pAT153 (12) and pBR322 containing the EcoRI J fragment of EBV DNA (13) were propagated in E.coli and DNA prepared as described previously (13).

c) Enzymes

Restriction endonucleases EcoRI and ClaI were purchased from Boehringer; AccI, SmaI, PvuII, MboII, HinfI and Sau 3AI were from New England Biolabs. BamHI was obtained from P.L. Biochemicals and SstI was prepared by standard methods (14). Conditions for endonuclease digestion and agarose gel electrophoresis were as described (15). RNase T1 was purchased from Calbiochem and S1 nuclease from Sigma. Calf intestinal alkaline phosphatase was obtained from Boehringer and phage T4 induced polynucleotide kinase from PL Biochemicals. T4 DNA polymerase was a gift from Dr. N. Smolar. Pancreatic DNase was purchased from Worthington and purified as described (10).

d) Construction of plasmids containing individual small RNA genes

Plasmid pBR322 containing the EBV EcoRI J fragment (pJ) was cleaved with EcoRI and SstI and the 1kb fragment which specifies the small RNAs (5) was separated by agarose gel electrophoresis. After isolation and purification this fragment was bisected by cleavage with Sau 3AI. The products were used in two cloning strategies: (A) ligation to pAT153 which had been cleaved with EcoRI + BamHI; (B) ClaI linearised pAT153 was added to the fragments and the mixture was treated with phage T4 DNA polymerase in the presence of all four deoxyribonucleoside triphosphates using conditions described previously (16). The flush ended products from this reaction were then ligated as described (5). The products of both ligations (A and B) were used to transform E.coli HB101. Plating and growth of bacteria followed by colony screening were all as described

previously (13).

e) In vitro transcription

HeLa and KB cells were grown to a density of approximately $5x10^5$ cells per ml in suspension culture in Joklik's medium supplemented with 10% foetal bovine serum and glutamine. Extracts were prepared from HeLa cells by the Manley et al. (17) procedure. KB cell or Raji cell (grown as in (a)) extracts were prepared according to Wu (18). All in vitro transcription reactions and purification of products were carried out using standard methods (18,19).

f) Nuclease S1 analysis

The method was based on the Weaver and Weissmann (20) variation of the Berk and Sharp procedure (21). Plasmid pJ was cleaved with EcoRI + SmaI and the fragments were 5'-end labelled using polynucleotide kinase and γ-^{32}P ATP (11) or 3'-end labelled with T4 DNA polymerase and α-^{32}P deoxyribonucleoside triphosphates (16). Complementary strands of these DNA probes were separated on 4% polyacrylamide strand separating gels (22). A portion of each labelled probe was retained for a purine-specific DNA sequencing reaction (22) whilst the remainder was annealed to samples of RNA synthesised either in vitro or in vivo and treated with S1 nuclease as described (10). S1 resistant DNA products were fractionated on an 8% polyacrylamide urea gel (23) alongside the corresponding DNA sequencing sample.

g) Hybridisation of labelled RNA to immobilised EBV DNA fragments

Gel fractionated restriction endonuclease fragments of EBV DNA were transferred to nitrocellulose membranes and hybridised with in vitro labelled cytoplasmic RNA from Raji cells or with labelled RNA synthesised in vitro from cloned EBV DNA fragment templates all as described previously (5).

RESULTS

In vitro synthesis of EBV specified small RNA

The restriction map of EBV EcoRI J (5) is shown in Figure 1. Previous data (5) demonstrated that the J-specific transcripts (J-RNAs) map within the rightmost one third of the fragment between the SstI site and the end. The DNA sequence (5,8) of part of this region is shown in Figure 2.

For in vitro transcription experiments, plasmid pBR322 containing the EBV EcoRI J fragment (pJ) was cleaved with various restriction

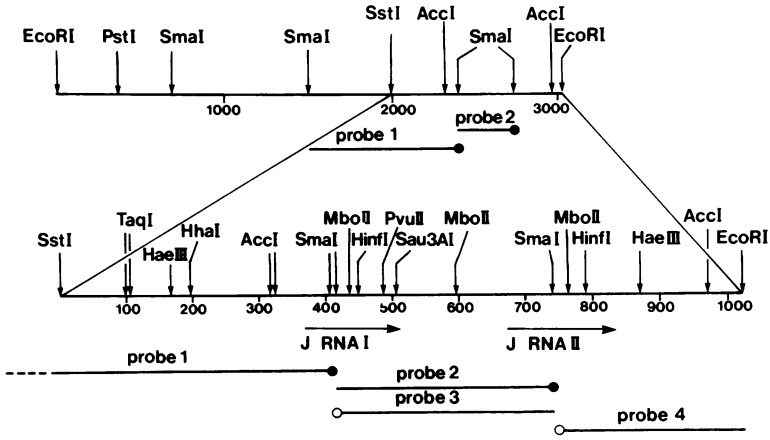

Figure 1
Restriction endonuclease map of the EBV EcoRI J fragment and fine structure map of the region which specifies the two small RNAs. The scales are in base pairs. The locations of the coding sequences and direction of transcription of the two J RNAs are indicated by the horizontal arrows. The fragments used as probes for determining the positions of the 5'-termini (probes 1 and 2) and 3'-termini (probes 3 and 4) of the J RNAs are indicated. • designates a 5' label and o a 3' label on the probes.

endonucleases and the resulting mixture of fragments (derived from both vector and insert) was used as template in the HeLa whole cell extract system (17) utilising the run-off assay (24). In vitro transcription of EcoRI cleaved pJ yielded specific, major products about 170-180 bases in length as judged by polyacrylamide gel electrophoresis (Figure 3). These RNAs were not synthesised in the absence of added DNA or if EcoRI cleaved pBR322 is used as template. Thus these RNA species must be transcribed from the EBV sequences present in the recombinant plasmid.

Synthesis of the small RNA is mediated by RNA polymerase III

To determine which form of RNA polymerase was responsible for this transcription, the experiment was repeated in the presence of various concentrations of α-amanitin. Synthesis of the small RNA species was unaffected by 10µg/ml α-amanitin but was inhibited by a concentration of 200µg/ml (Figure 3). This result indicates that the J-RNAs are synthesised by RNA polymerase III (25).

Nuclease S1 mapping of the termini of in vitro and in vivo synthesised small RNAs

In order th map precisely the termini of the small RNA products,

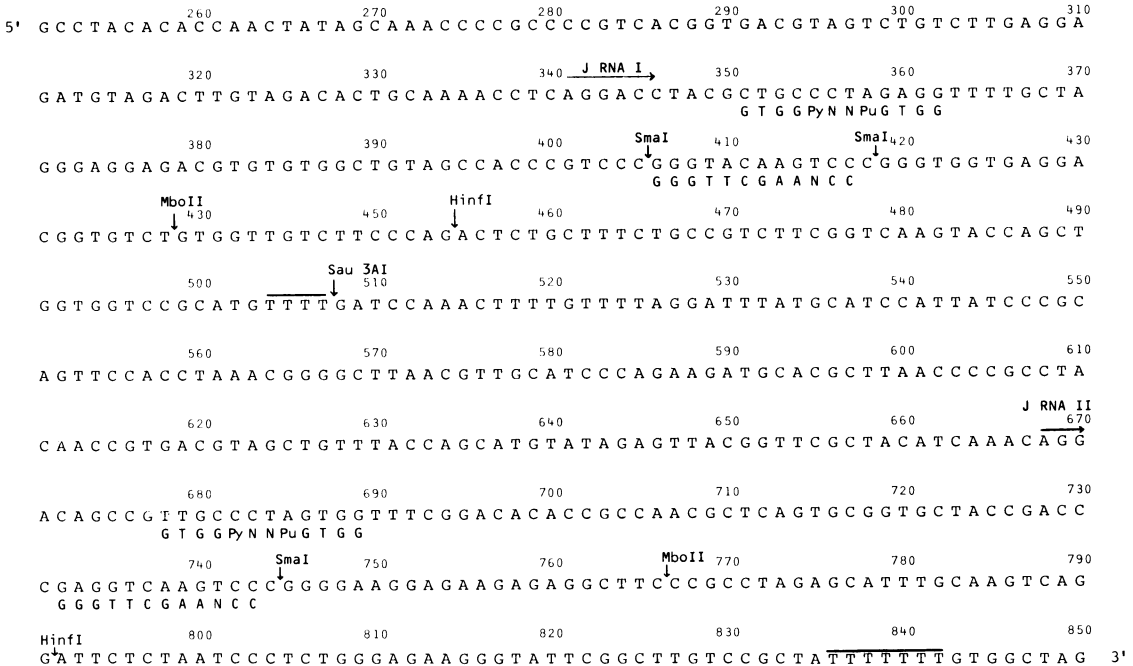

Figure 2 The DNA sequence of that part of the EBV EcoRI J fragment which specifies the small RNAs. Only the strand homologous to the major RNAs is shown. The numbering system is that of Arrand and Rymo (5) which starts at the SstI cleavage site (see Figure 1) on the DNA strand shown here. The sequence has been revised from that reported earlier (5) by the inclusion of an extra GT at positions 383-384 and extra C residues at positions 399 and 803. The 5'-ends and direction of transcription of J RNAI and J RNAII are indicated by arrows and the corresponding termination T-clusters are overlined. The cleavage sites of several restriction endonucleases referred to in the text are marked. The consensus sequences for RNA polymerase III intragenic control regions (27) are shown in their appropriate positions below the sequence (nucleotides 351-362, 406-417, 678-689 and 732-743).

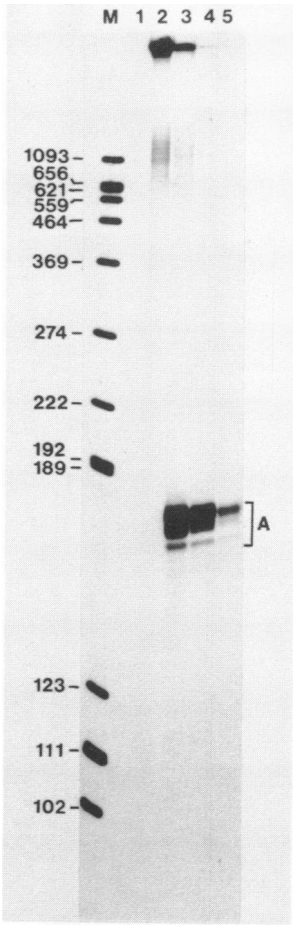

Figure 3
Analysis of ^{32}P-labelled RNA synthesised *in vitro*. Polyacrylamide urea gel fractionation of α-^{32}P-GTP labelled *in vitro* run-off products synthesised using HeLa cell extracts with no added template (1) *Eco*RI cleaved pBR322 (2), *Eco*RI cleaved pJ in the absence of α-amanitin (3) or the presence of 10μg/ml (4) and 200μg/ml (5) α-amanitin. Lane "M" contains ^{32}P-labelled DNA size markers, the lengths of which are indicated to the left of the autoradiogram. The EBV DNA-specific products of lengths 170-180 nucleotides are indicated "A".

unlabelled RNA synthesised *in vitro* was hybridised to 5' or 3'-end labelled, separated strands of the *Eco*RI + *Sma*I double digest fragments of pJ (see Figure 1). After annealing, the hybrids were treated with S1 nuclease, denatured and run on an 8% polyacrylamide gel containing 7M urea.

S1 resistant hybrids were obtained only with the fragments (probes 1-4) indicated in Figure 1. None of the other fragments produced hybrids which contained label. A purine specific sequencing track (22) of the corresponding DNA fragment was run alongside each sample. Since the DNA sequence of this part of the DNA is known (5,8), it was possible to align the S1 trimmed DNA fragment with the DNA sequence and thus define precisely the 5' and 3' termini of two small RNAs. The results are shown in Figures 4 and 5 and are identical using both *in vitro* and *in vivo* RNA. Thus the *in vitro* transcription system both initiates and terminates RNA synthesis with good fidelity. The two 5' termini lie at positions 341±2 and 669±2 in the DNA sequence and the 3' ends are at positions 504±2 and 836±2 (see Figure 2).

Effect of further restriction endoclease cleavage of the template

*Eco*RI cleaved plasmid pJ was further cleaved with *Sst*I, *Acc*I, *Pvu*II or *Sma*I (see Figure 1) and the doubly cleaved DNA was used as template in the *in vitro* transcription system. The results are shown in Figure 6. As anticipated, secondary cleavage with *Sst*I which cuts well outside the transcribed regions had no effect. More unexpectedly *Acc*I, which cuts 15 nucleotides upstream from the initiation point on the template strand of J RNA gene I (see Figures 1 and 2) and which may be expected to influence synthesis of this RNA, also had very little or no effect. Similarly, cleavage with *Eco*RI + *Pvu*II which would have been expected to yield a truncated product from gene I, produced no visible change in the *in vitro* products when analysed by direct polyacrylamide gel electrophoresis. Conversely, instead of leading to the synthesis of truncated products, secondary cleavage of the template with *Sma*I completely abolished transcription of the small RNAs. These results suggested that (i) *in vitro* synthesis of RNA from gene II vastly exceeded that of gene I and (ii) the *Sma*I site in gene II may lie within an important intragenic transcriptional control region.

The first suggestion was verified by eluting the major gel fractionated products from an *in vitro* transcription reaction which used *Eco*RI cleaved pJ as template and hybridising to DNA from coliphage M13 which contained cloned segments of each transcribed region (5). Hybridisation was observed only to those phage DNAs which contained the coding strand of gene II (data not shown).

Separation of the two small RNA genes into different plasmids and transcription in vitro

The foregoing result suggested that competition may exist between the

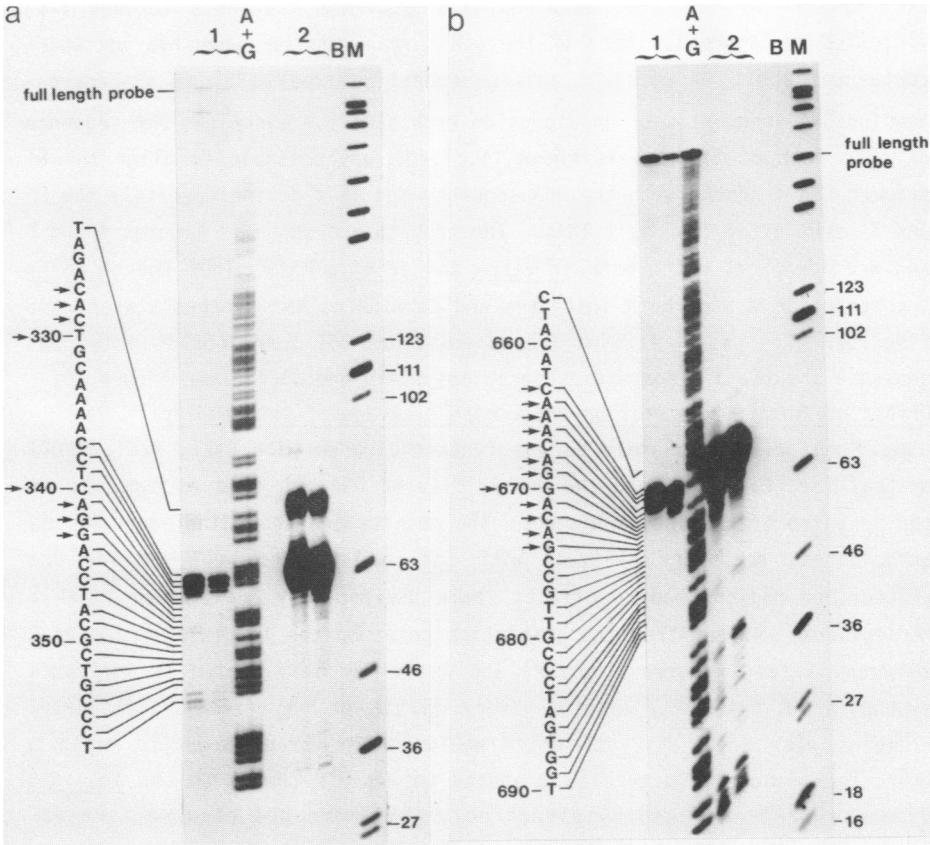

Figure 4 Localisation of the 5'-ends of the J RNAs by S1 nuclease gel mapping. The hybridisations contained approximately 2ng of 5'-^{32}P-labelled single stranded DNA fragments (Probe 1, labelled at position 405 (panel a) or Probe 2, labelled at position 744 (panel b); see Figure 1) and the following RNA samples: tracks 1, RNA synthesised in vitro from 37.5µg/ml of EcoRI cleaved pJ corresponding to 20µl (lefthand tracks of each pair) and 10µl (righthand tracks) incubations; tracks 2, 10µg (lefthand track of each pair) and 2.5µg of cytoplasmic RNA extracted from Raji cells; B, carrier RNA alone. Tracks "A+G" are markers generated by purine-specific cleavage of the hybridisation probes. The correspondence between bands on the autoradiogram and the deduced position on the DNA sequence is shown. These autoradiographs have been deliberately overexposed so that the sequencing lanes are clearly visible on the photographs. The precise alignment of the S1-resistant hybrids with the DNA sequencing lanes was done on a much lighter exposure of the autoradiograph. On this light exposure the minor, slower migrating S1-resistant hybrid observed at position 329±2 in panel a was visually estimated to represent less than 1% of the major species. It may indicate an additional, minor 5'-end found only in vivo or it could be an artifact of hybridisation in RNA excess. This latter possibility is considered likely since under conditions of DNA excess this band is not observed.

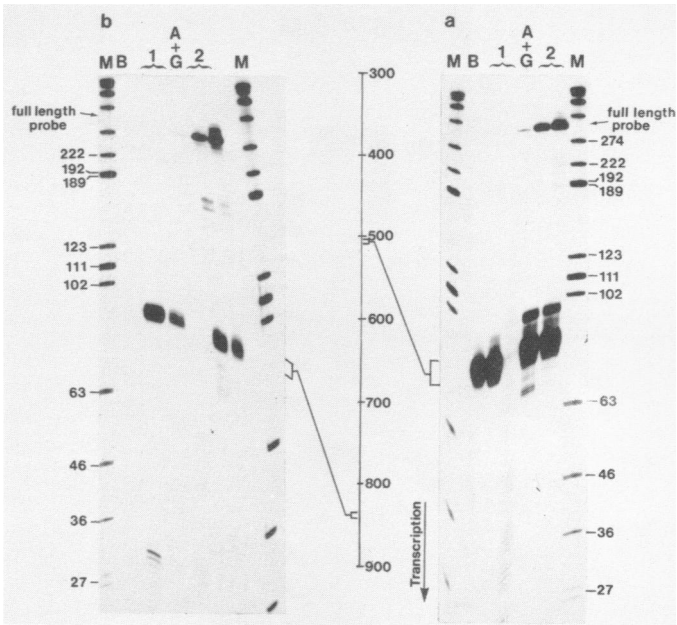

Figure 5

High resolution mapping of the 3' termini of the J RNAs by S1 analysis. The hybridisations (as in Figure 4) contained 3'-^{32}P-labelled single stranded DNA fragments (probe 3, labelled at position 419 (panel a) or probe 4, labelled at position 745 (panel b); see Figure 1) and the following RNA samples: B, carrier RNA alone; tracks 1, 5µg (lefthand tracks of each pair) and 10µg (righthand tracks) of cytoplasmic RNA extracted from Raji cells; tracks 2, RNA synthesised *in vitro* equivalent to 15µl (lefthand tracks of each pair) and 30µl (righthand tracks) from 37.5µg/ml of *Eco*RI cleaved pJ. Tracks "A+G" are purine-specific cleavages of the hybridisation probes. The position of the 3'-ends as deduced by size determination and alignment on the DNA sequence is indicated. The minor apparent 3' terminus detected only with *in vitro* RNA (panel a) probably corresponds to ends of RNA chains which had not terminated at the principal termination site within the thymidine cluster at positions 504-507 and had read through to terminate within the thymidine cluster at positions 517-520. This could imply that the crude HeLa whole cell extract is somewhat deficient in a component required for efficient termination by RNA polymerase III. However the apparent minor 3'-end could also represent an S1 artefact.

two small RNA genes and that the affinity of RNA polymerase III and/or transcriptional factors for the gene II promoter could be much greater than for the gene I promoter. By making use of *Sau* 3AI which cleaves between the two genes, the two transcriptional regions were separately cloned in different plasmids as detailed in the methods section. By virtue of the DNA sequence to the right of the *Sau* 3AI site (GATCC, see Figure 2),

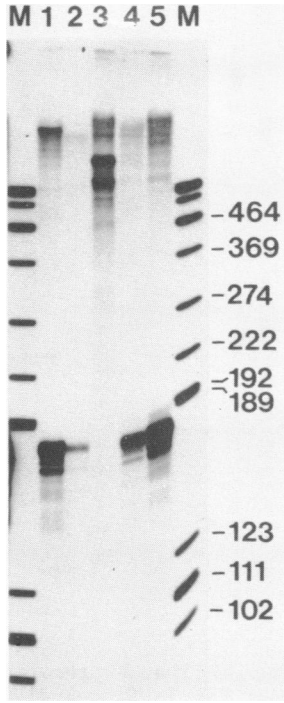

Figure 6
Effects on *in vitro* transcription of cleavage of the template DNA with various restriction endonucleases. Experimental conditions and size markers (M) were as in Figure 3. The DNA templates were plasmid pJ cleaved with EcoRI + PvuII (1), EcoRI + AccI (2), EcoRI + SmaI (3), EcoRI + SstI (4), EcoRI (5). The apparent inhibition of RNA synthesis following AccI cleavage (lane 2) is not characteristic and is due to manipulative losses in this particular experiment.

ligation of the Sau 3AI - EcoRI fragment (nucleotide 508 to the right end of EcoRI J, see Figures 1 and 2) into EcoRI + BamHI cleaved plasmid vector regenerated both the BamHI and EcoRI sites allowing this cloned fragment to be conveniently recovered from the recombinant.

The structures of the recombinant plasmids were verified by digestion with various restriction endonucleases followed by agarose gel electrophoretic analysis of the products and by DNA sequence analysis of the termini of the inserted fragments.

The recombinant plasmid containing J RNA gene I was obtained by blunt end ligation into the ClaI site of the vector and is referred to as pJJJ1. The plasmid which contains gene II was obtained by ligation into EcoRI +

BamHI cleaved pAT153 and is called pJJJ2.

When these plasmids were used as templates for in vitro transcription the results were identical to those obtained when the two genes were present on the same plasmid i.e. gene I yielded very little product whereas gene II was efficiently transcribed (Figure 7). This result is in contrast

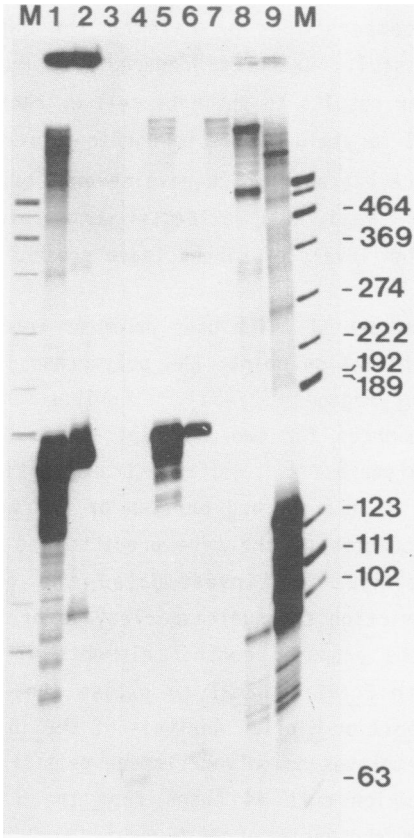

Figure 7

In vitro transcription of J RNAs by HeLa and KB cell extracts using DNA templates containing separated J RNA genes. Experimental conditions and size markers were as in Figure 3. Templates and extracts were: lane 1, pJ cleaved with EcoRI, HeLa cell extract; lane 2, as 1, KB cell extract; lane 3, pJJJ1 cleaved with EcoRI + BamHI, HeLa cell extract; lane 4, as 3, KB cell extract; lane 5, pJJJ2 cleaved with EcoRI + BamHI, HeLa cell extract; lane 6, as 5, KB cell extract; lane 7, pJJJ2 cleaved with EcoRI + SmaI, HeLa cell extract; lane 8, pJJJ2 cleaved with EcoRI + BamHI + MboII, HeLa cell extract; lane 9, pJJJ2 cleaved with EcoRI + BamHI + HinfI, HeLa cell extract.

to the S1 mapping data which did not detect a difference in transcriptional efficiency between J RNA I and J RNA II (Figures 4 and 5). This is because the S1 mapping experiments were oversensitive due to the hybridisations being carried out under conditions of RNA excess. Such experiments establish only that both genes are active but do not comment on their relative efficiencies.

Attempts to increase the amount of gene I transcription by using various incubation temperatures or raising the DNA concentration to 225µg/ml were unsuccessful. Extracts prepared from Raji cells or from KB cells (18) gave similar results to the HeLa cell extracts. However, the KB cell extracts appeared to yield a more homogeneous product than that which was obtained using HeLa extracts which gave several prematurely terminated species (Figure 7). Raji cell extracts were relatively inefficient probably due to the high levels of ribonuclease present in lymphocytes (26) (data not shown).

Unlike RNA polymerase II which uses promoter sequences upstream from the transcriptional initiation point, RNA polymerase III is influenced by intragenic controlling regions (27-31). Fowlkes and Shenk (29) have derived consensus sequences for two such intragenic elements involved in the control of RNA polymerase III mediated transcription. Examination of the DNA sequence of the transcribed portion of EBV EcoRI J reveals that both genes contain sequences of the type predicted to be such controlling regions (see Figure 2). We investigated the effect on *in vitro* transcription of restriction endonuclease cleavage of the template in the vicinity of one of the proposed control elements within J RNA gene II. pJJJ2 was cleaved with EcoRI + BamHI to excise the insert, followed by cleavage with SmaI, MboII or HinfI. Analysis of the *in vitro* transcription products from these templates on polyacrylamide gels (Figure 7) showed that cleavage with HinfI which cuts 44 bases from the 3' end of the coding sequence leads to the synthesis of a truncated product of the expected size; MboII cleavage 23 bases 3' from the end of the consensus control region produces an inefficient template which yields a low level of truncated product, whereas cleavage with SmaI which cleaves immediately adjacent to the predicted control region (Figure 2) completely abolishes RNA synthesis, in agreement with the proposed importance of this region. The marked repression of the level of RNA synthesis following MboII cleavage of the template suggests that the intragenic control region may extend some distance 3' from the consensus sequence.

Estimation of relative amounts of the two J RNAs in vivo

The disparate levels of in vitro synthesis of the two J RNAs prompted us to examine the relative levels of the two species found in vivo. Plasmid pJJJ1 was cleaved with EcoRI and HindIII, pJJJ2 was cleaved with EcoRI and BamHI whilst pJ was cleaved with EcoRI and SstI. Following agarose gel electrophoresis the resulting DNA fragments were transferred to nitrocellulose membranes and hybridised with cytoplasmic RNA from Raji cells which had been labelled in vitro with ^{32}P or with labelled RNA which had been synthesised in vitro using pJ or pJJJ2 as template. The results are shown in Figure 8. As expected, RNA synthesised in vitro using pJJJ2 as template hybridised strongly to DNA containing the J RNA II gene and showed very little or no cross hybridisation with pJJJ1. In keeping with previous data, RNA synthesised in vitro using pJ as template hybridised strongly to pJJJ2 but very weakly to pJJJ1. In contrast, cytoplasmic RNA from Raji cells hybridised strongly to pJJJ1 and weakly to pJJJ2 indicating that in vivo the abundance of J RNA I greatly exceeds that of J RNA II.

DISCUSSION

In this work we have shown that cloned DNA from the EcoRI J fragment of the EBV genome can be used as template in a cell-free system for the synthesis in vitro of two small RNAs. Nuclease S1 mapping studies of the in vitro transcribed products and of cytoplasmic RNA prepared from Raji cells has located the positions of these RNAs precisely within the DNA sequence of this region of the EBV genome. Further, it has shown that in vitro initiation and termination of transcription of these two molecules is a faithful parallel of the in vivo situation. However, the relative level of transcription of the two RNAs in vitro is different from that observed in vivo. Transcription of the EBV specified small RNAs is mediated by RNA polymerase III as demonstrated by its sensitivity to varying levels of the toxin α-amanitin. Potential intragenic controlling sequences have been identified within both J RNA genes.

Control of RNA polymerase III mediated transcription involves not only the intragenic control regions but also in some cases, e.g. tRNA genes (33,34) their 5' flanking sequences. However in other instances, e.g. Xenopus 5S RNA this region appears to be unnecessary (27). It has been suggested (27) that the intragenic control region specifies the approximate transcriptional startpoint, perhaps by some measuring process, and that the

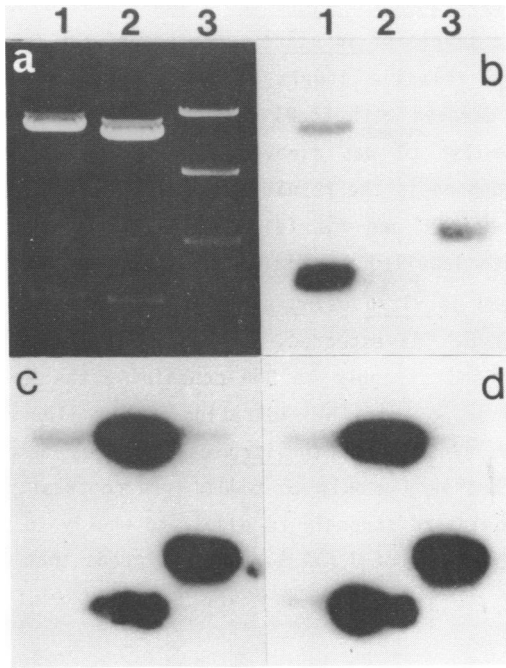

Figure 8

Comparison of the relative amounts of J RNA I and J RNA II *in vivo* and *in vitro*. Fragments of DNA containing the J RNA genes were excised by cleaving plasmid pJJ1 with EcoRI plus Hind III (lanes 1), plasmid pJJ2 with EcoRI plus BamHI (lanes 2) and plasmid pJ with EcoRI plus SstI (lanes 3). The products were separated by electrophoresis on 0.8% agarose gels. Panel A shows the pattern of fragments after ethidium bromide staining and visualisation under u.v. light. Lanes 1 and 2 contain some partial digestion products (i.e. cleaved by only one of the two enzymes) leading to the doublet appearance of the slow moving fragment. DNA was transferred to nitrocellulose membranes and hybridised with Raji cell cytoplasmic RNA which had been labelled *in vitro* with ^{32}P (Panel B) or with ^{32}P-labelled RNA synthesised *in vitro*. 30µg of pJJ2 or pJ were incubated in a 500µl standard transcription reaction containing 500µCi ^{32}P-GTP. The products were fractionated on an 8% polyacrylamide urea gel and the EBV-specified RNAs were eluted and used for hybridisation. Panels C and D show respectively the patterns of hybridisation obtained with RNA synthesised using pJJ2 and pJ as template.

exact initiation point is determined by surrounding sequences in the 5' flanking region. Examination of the J RNA gene flanking sequences show substantial homology between the sequences immediately upstream of the 5'-ends (Figure 9) but no significant homology with the 5' flanking regions of other RNA polymerase III transcribed genes. In particular, the

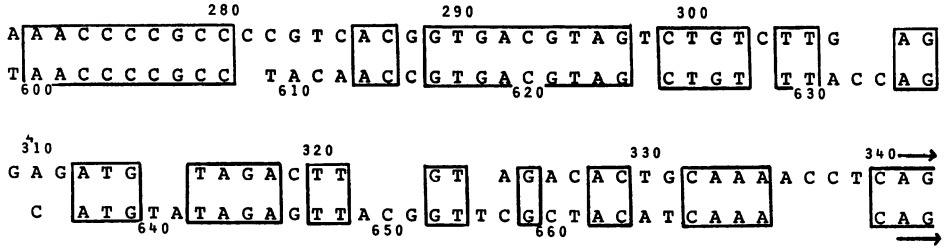

Figure 9

Comparison of the DNA sequences flanking the 5'-ends of the two J RNA genes. The sequences are numbered as in Figure 2 and are aligned leaving gaps where necessary to maximise homology. The initiation points of the two RNAs are indicated by arrows.

canonical sequences postulated by Korn and Brown (35) to be potential control elements in the transcription of various Xenopus and Drosophila 5S RNAs and Ad2 VA RNA_I and which are also found upstream of the Ad2 VA RNA_{II} (36), are not found in the EBV system. Repeat sequences have been observed in the 5' flanking regions of several polymerase III transcribed genes (35,36). The J RNA I gene is prefaced by a TGTAGAC repeat (AccI site) but the J RNA II gene does not have any significant flanking repeat units.

It has been pointed out that RNA polymerase III mediated transcription terminates at a cluster of four or more T residues on the non-coding strand (35). The two J RNA genes are no exception; the first gene terminates at a T_4 cluster whilst the second ends at a T_7 (Figure 2). Bogenhagen and Brown (37) remarked that the T cluster alone is not sufficient for termination since some genes e.g. Ad2 VA RNA_{II} (36) and some tRNAs (38,39) contain internal T clusters. J RNA I provides another example, containing an internal cluster of 4 T residues at nucleotide positions 363-366 (Figure 2) which is 23 to 26 residues from the 5'-end of the RNA. Clusters of 5 or more T residues appear to terminate well regardless of their surrounding sequences whereas T_4 clusters seem to function as efficient termination signals when surrounded by G and C residues. They work poorly when flanked by A-rich sequences (37). In keeping with this observation the T_4 cluster at the termination point of the J RNA I gene is flanked by GC rich sequences but the internal T_4 is devoid of surrounding C residues and approximates to one of the very weak termination sequences demonstrated by Bogenhagen and Brown (37).

Cells infected with adenovirus-2 contain widely differing levels of

the two VA RNAs and this disparity is reflected when these two genes are used as transcriptional templates in vitro (29,20,40). Raji cells also appear to contain different levels of the two J RNAs (Figure 8). However our experiments using the HeLa whole cell extract (17) in vitro transcription system led to widely different levels of synthesis of the two species but the relative amounts were reversed as compared with the in vivo situation. On the other hand, Rosa et al. (8) using a cytoplasmic extract prepared from KB cells (18) seemed to obtain comparable levels of the two EBV RNAs in vitro. This latter discrepancy at first seemed likely to be due to the use of different cell-free systems since it had been observed (40) that extracts from different cell types differed in their ability to discriminate between the two VA RNA genes. However we have been unable to confirm the result of Rosa et al. (8). In our hands a KB cell cytoplasmic S20 extract (18) or S100 extract (40) gave identical results to those obtained using the HeLa whole cell extract (see Figure 7).

A further possibility was that a lymphocyte-specific factor(s) may be required for efficient transcription of the J RNA I gene. However this does not seem to be the case since an S20 extract prepared from Raji cells gave similar results to those obtained using HeLa or KB cell extracts (data not shown).

The basis of the differential selectivity of J RNA transcription in vitro and in vivo is at present unclear. We have drawn attention to the similarities between the two genes in various areas which may be anticipated to affect control of transcription, i.e. their 5' flanking sequences (Figure 9), homologous 5' termini (Figure 2 and refs. 5, 8), intragenic controlling sequences (Figure 2) and overall internal sequence homology (5). In view of these great similarities the large difference in efficiency of in vitro transcription is somewhat surprising. However our experiments rule out the possibility of competition between the genes for limiting polymerase and/or factors required for transcription, since templates containing the genes in isolation behave in the same manner as templates which contain the two linked genes. There remains the possibility that J RNA I transcription in vitro requires the presence of a labile factor(s) which is lost during preparation of the cell-free extracts.

A second strange feature emerging from this work is the reversal in the relative levels of the two RNA species in vivo and in vitro. Although it appears that J RNA II is produced in vitro more efficiently than J RNA I

it is possible that in vivo the rate of turnover of J RNA II significantly exceeds that of J RNA I. This could then lead to the observed steady state situation where the amount of J RNA I exceeds that of J RNA II. Pulse labelling studies could be employed to resolve this question.

The function(s) of the small, nonpolyadenylated J RNAs is a matter for conjecture. It has been suggested (8) that in view of the many parallels that can be drawn between the EBV J RNAs and the Ad2 VA RNAs they may assume a common function in the two viral systems. A hypothesis has been put forward that the VA RNAs could be involved in the splicing of adenovirus mRNAs (41) and this notion has some experimental support (42,43). An alternative suggestion (42) was that VA RNAs may have a role in the transport of processed messengers from nucleus to cytoplasm.

More recently it was suggested (44) that since interaction between some small RNAs and ribosomes (45,46) or mRNAs (47) has been demonstrated, it is possible that VA RNAs and, by analogy, the EBV J RNAs may have a role in translation. Base pairing between the 3'-end of 18S ribosomal RNA and specific sequences on mRNA has been proposed as a basis for the correct positioning of ribosomes on messages during initiation of translation (48). Ad2 VAI RNA could form similar association (44) although the sequence of the VAII species does not allow an analogous interaction. Interestingly, one of the highly conserved regions within both of the EBV J RNAs (5) could form a similar complex. This relationship, which is as extensive as that proposed for some mRNA-rRNA associations (48), is shown in Figure 10 and may account for Rymo's original observation (1) of high levels of J RNAs associated with polyribosomes. Many more studies are obviously necessary to determine unequivocally the function of these highly abundant, viral-specified RNAs.

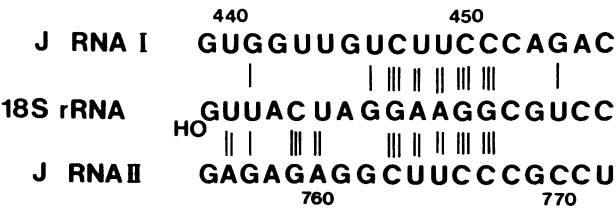

Figure 10
Partial complementarity between the J RNAs and the 3'-end of 18S rRNA. The numbering is as in Figure 2. Note that the sequence showing the greatest complementarity is perfectly conserved within the primary structure of both J RNAs.

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