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CMER, an RNA encoded by human cytomegalovirus is most likely transcribed by RNA polymerase III

Rolf Marschalek, Elfi Amon-Böhm, Jay Stoerker[1+], Sabine Klages[1], Bernhard Fleckenstein[1] and Theodor Dingermann*

Institute für Biochemie der Medizinischen Fakultät, Universität Erlangen-Nürnberg, Fahrstraße 17, D-8520 Erlangen and [1]Institut für klinische und molekulare Virologie, Universität Erlangen Nürnberg, Loschgestraße 7, D-8520 Erlangen, FRG

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ABSTRACT

Through computer analysis of a human cytomegalovirus (HCMV) genomic region, previously identified to be homologous to human genomic DNA, an element showing significant similarity to the 3'-internal control region (3'-ICR or B-block) of a eukaryotic RNA polymerase III promoter could be detected. This region - located on the EcoRI b fragment within the UL segment of the viral genome of HCMV strain AD 169 - cannot be transcribed *in vitro* in an RNA polymerase III specific transcription system. However, this part of the viral genome is able to compete for components of the RNA polymerase III transcription complex as shown in template exclusion experiments and by gel retardation assays. Two different synthetic oligonucleotides complementary to the 3'-ICR and to nucleotides located immediately downstream of this promoter element can anneal specifically to a HCMV-encoded ribonucleic acid (termed CMER) synthesized in human foreskin fibroblasts (HFF) late in virus replication. As a consequence of identifying the transcription initiation point by primer extension analyses the position of the 5'-internal control region (5'-ICR or A-block) of the CMER gene could be uncovered. Both identified control regions (the A-block as well as the B-block) of the transcription unit exhibit significant similarities to corresponding regulatory elements of other class III genes, including virus encoded class III genes. Initiation of *in vivo* transcription occurs 15 nucleotides upstream of the 5'-border of the 5'-ICR and the two non-contiguous gene internal promoter elements are separated by 79 nucleotides.

INTRODUCTION

Small RNAs encoded by viral genomes have been reported for a variety of systems. Two RNA genes (VAI and VAII) are located at about 30 map units on the adenovirus type 2 or 5 (Ad2 or Ad5) chromosome (1, 2). Two genes coding for small RNAs (EBER 1 and EBER 2) have been identified on the 3 kbp EcoRI-J fragment at the left end of the Epstein-Barr virus (EBV) genome (3). Finally, coding capacity for four different small RNAs was detected on the left-most 7.4 kbp of the L-DNA from *Herpesvirus* (*H.*) *saimiri* (4). At least in case of VAI and VAII (5, 6) as well as EBER 1 and EBER 2 (7, 8) it was demonstrated that the RNAs are transcribed by RNA polymerase III. Characteristic for those genes is the unusual location of their promoters: class III genes comprise gene-internal promoters consisting of two non-contiguous control regions termed ICRs (internal control regions) or A-block and B-block (9 - 13). Specific transcription factors are sequestered onto these ICRs which remain stably bound for many rounds of transcription (14, 15). In case of 5S rRNA synthesis three transcription factors, TFIIIA, TFIIIB and TFIIIC are involved in complex formation, while transcription complexes on tRNA genes as well as on virus associated genes contain factors TFIIIB and TFIIIC (16). RNA polymerase III, finally, associates with stable transcription complexes and initiates transcription.

We were interested in whether HCMV — a herpesvirus like Epstein-Barr virus and *H. saimiri* — also comprise genes coding for small RNAs. Genomic size and complexity of HCMV are considerably larger than

those of most other human herpesviruses (6, 18); the genome of HCMV is a linear duplex DNA molecule of about 235 kbp. The genome can be subdivided into a large (L) and a small (S) segment (19, 20). The L segment of HCMV strain AD169 consists of a unique region of 174 kbp (U_L) which is flanked by short inverted repeats of 10.6 kbp (IR_L/ TR_L). Similar inverted repeats of 2.4 kbp (IR_S/TR_S) flank the 35.6 kbp long S segment (U_S). U_S and U_L may be oriented in either directions, resulting in four isomeric forms of the viral genome, which occur in about equal proportions. The complete viral genome of HCMV has been cloned into cosmid vectors (21) and into plasmids (22, 23) and physical maps have been constructed (24) (see Figure 1).

Here we present results demonstrating that a virus associated RNA (termed cytomegalovirus encoded RNA or CMER) is synthesized in human foreskin fibroblast cells late after infection. Several lines of evidence led to the conclusion that the corresponding gene, located on the EcoRI b-fragment within the U_L region of HCMV AD169, is transcribed by RNA polymerase III.

MATERIALS AND METHODS

Cloning of the HCMV genome.

Construction and characterization of a cosmid library representing the entire HCMV genome has been described (21). A variety of subclones was generated by inserting DNA fragments into plasmid vectors pACYC184 (25), pHC79 (26), and pBR322 or pUC18, using standard methods. pRR8 contains a 2.8 kb EcoRI/HindIII fragment derived from cosmid pCM1017 ligated into pBR322. pUC379 is a derivative of pRR8 comprising an AsuI fragment inserted into pUC18 after BamH1 linker ligation. The relative position of relevant clones used in this study are outlined in Figure 1.

In vitro transcription and template exclusion assays.

Cellular extracts with specific RNA polymerase III transcription activity were prepared from HeLa cells according to Weil et al. (27). Transcription assays were carried out at room temperature in a total volume of 40µl containing 0.5µg plasmid DNA, each 0.5mM of ATP, CTP and GTP, 0.05M UTP, 3µCi [5–^{3}H]UTP (19.1 Ci/mmol), 0.5mM dithiothreitol, 30mM Hepes pH 8.0, 100mM KCl, 5mM $MgCl_2$ and 20µl HeLa extract. Reactions were terminated by a 15-min proteinase K (30µg) digestion, phenol extraction and ethanol precipitation. Transcription products were separated on 8% polyacrylamide gels (acrylamide/bisacrylamide weight ration 20:1) containing 50% urea and 100mM Tris/borate pH 9.2, 2mM EDTA (28). Gels were treated with dimethyl sulfoxide/2,5-diphenyloxazole (29), dried and exposed to X-ray film at -80°C.

For template exclusion assays 0.3µg of plasmid pArg was added 15 min after initiation of transcription by the addition of HeLa extract (15). pArg consists of a 508 bp HindIII genomic *Drosophila melanogaster* DNA fragment carrying the gene for the major $tRNA^{Arg}$ species inserted into the HindIII site of pBR322 (30). Assays were incubated for further 60 min after addition of competitor plasmid and then processed as described above.

Gel retention assays.

Gel retention assays were performed as described by Strauss et al. (31) with the following modifications: the total assay volume was 15µl and randomly sheared *E. coli* DNA or poly(dI-dC) DNA were used as unspecific competitors. DNA fragments were labeled by a "fill in" reaction with [α^{32}P]dATP and DNA polymerase (Klenow fragment). Labeled fragments were purified on 8% polyacrylamide gels (acrylamide/bisacrylamide weight ration 30:1).

One ng ^{32}P labeled DNA fragment, unspecific competitor DNA (0.19µg - 20.0µg) and HeLa extract were incubated with 0.1% Triton X-100, 4% glycerol, 1mM Na-EDTA, 10mM Tris/HCl pH 7.5. After incubation for 30 min at room temperature the reactions were loaded on a low-ionic-strength 4% polyacrylamide gel containing 1mM Na-EDTA, 3.3mM Na-acetate, 6.7mM Tris/HCl pH 7.5 (31). The gel was pre-run for 1 h at 20mA. Electrophoresis was carried out at 25mA for 2 - 3 h at 4°C. Gels were dried on a slab gel dryer and exposed to X-ray film in the presence of enhancing screens at -80°C.

Isolation of small cellular RNA.

Mock-infected human foreskin fibroblast cells (HFF cells) and cells infected with HCMV were collected after treatment with trypsin, washed, and quickly resuspended in TMS buffer (10mM Tris/HCl pH 7.5, 10mM $MgCl_2$, 2mM $Na_2S_2O_3$). Na-dodecylsulfate was added to a final concentration of 0.5% and RNA was extracted twice with buffer saturated phenol at 4°C. The aqueous phase was passed through a DEAE-cellulose column equilibrated with TMS buffer. After washing the column with 0.2M NaCl in TMS buffer, RNAs were eluted with 0.7M NaCl in TMS buffer and precipitated with two volumes of ethanol/2% K-acetate at -20°C (32).

"Immediate early RNA" was prepared from cells grown in the presence of 100µg/ml cyclohexamide for 20 h after infection with at least 2 plaque forming units per cell, while "early RNA" was isolated from infected cells which were grown in medium containing 200µg/ml phosphonoacetic acid for 24 h. "Late" RNA was extracted 6 days after inoculation of cell cultures.

Primer extension analysis.

Five µg RNA, prepared from HCMV infected and from mock-infected HFF cells were coprecipitated with 10 pmol of a 5'-labeled primer fragment (1×10^5 - 1×10^6 Cerenkov cpm). Two different synthetic oligonucleotides were used as primer. Sequences and relative positions of complementarity of both primers within the CMER gene are depicted in Figure 2. The dry pellet was dissolved in 10 ml buffer containing 50mM KCl, 50mM Tris/HCl pH 8, and incubated at 90°C for 5 min and for 10 min at 0°C and at room temperature, respectively. Five ml of a buffer was added containing 50mM Tris/HCl pH 8, 50mM KCl, 20mM $MgCl_2$, 6mM DTT, 2.5mM dNTPs, and 3U AMV reverse transcriptase. Assays were incubated at 37°C for one hour and terminated by ethanol precipitation. The pellets were dissolved in 1.5 ml sterile distilled water and 2 ml loading buffer (95% formamide, 10mM EDTA, 0.1% bromphenol blue, 0.1% Xylene cyanol). Samples were boiled for 5 min, chilled on ice and products were separated on 8% polyacrylamide gels containing 50% urea (33).

A sequence analysis of a recombinant M13mp8 ss-DNA applying the dideoxy termination method (34) served as size marker. The universal sequencing primer (pentadecamer, New England Biolabs) was phosphorylated at its 5'-end prior to annealing onto the ss-DNA. This ensured identical electrophoretic mobilities of fragments generated during sequencing and in primer extension analyses.

RESULTS

A motif exhibiting significant similarity with a 3'-ICR of an eukaryotic class III promoter is located on the EcoRI b fragment of the HCMV genome.

Parts of the Human Cytomegalovirus (HCMV) exhibit sequence similarities to the cellular genome as deduced from hybridization experiments under stringent conditions (35, 36). In course of a computer search for functional

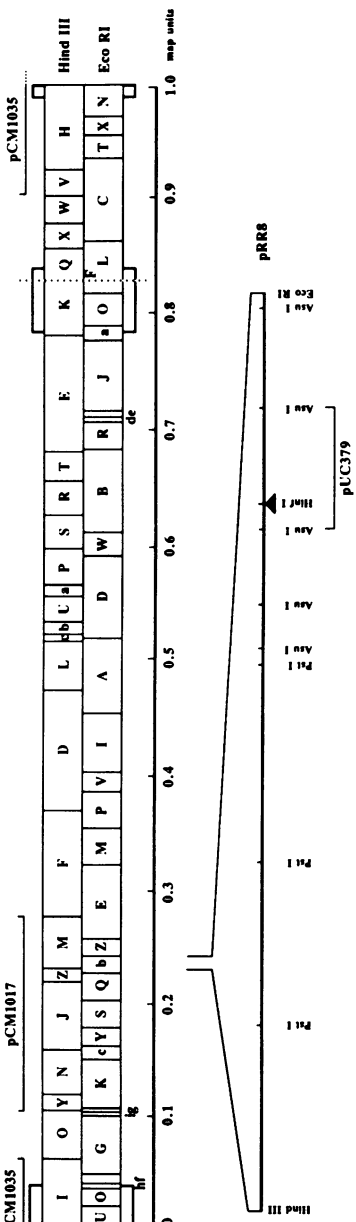

Figure 1. Physical map of the HCMV genome. Plasmids used in this study are indicated. pCM1035 and pCM1017 are cosmid clones. The EcoRI b fragment is part of pCM1017. A HindIII/EcoRI subfragment of this portion of the viral genome was recloned as plasmid pRR8. Subclone pUC379 was obtained after AsuI digestion followed by BamHI linker ligation. The putative "B-box"-like element is located on a 59 bp BamHI/HinfI subfragment of pUC379.

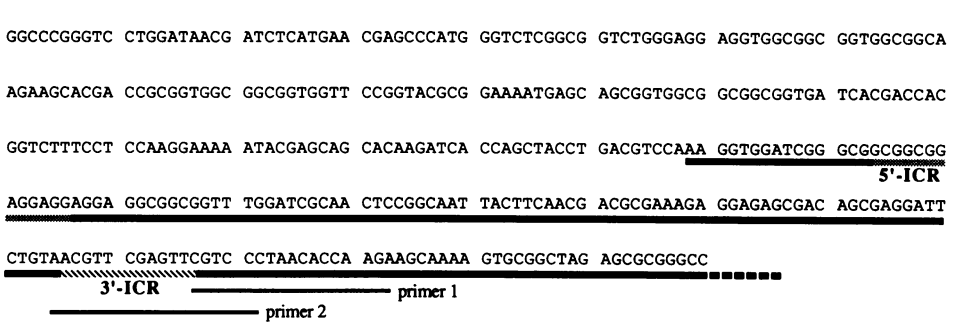

Figure 2. Nucleotide sequence of the BamH1 insert DNA from pUC379 (43). The coding region for CMER is underlined. Internal control regions (ICRs) determined based on extraordinary sequence similarity to the established ICR consensus sequences (see Figure 6) are indicated. The synthesis of cDNA from *in vivo* transcribed CMER (see Figure 5) was primed with synthetic oligonucleotides complementary to the region immediately downstream of the 3'-ICR (primer 1) and to parts of the 3'-ICR (primer 2).

elements located on hybridizing HCMV fragments a motif was detected on the EcoRI b fragment (see Figure 1), showing striking similarities to the B-box element (3'-ICR) of the eukaryotic class III gene promoter (13). In order to further investigate the functional relevance of this element, subclones pRR8 and pUC379 of the original cosmid pCM1017 were constructed (see Figure 1). These clones were assayed for *in vitro* transcription activity; however, no specific products are synthesized from this region during *in vitro* transcription neither in an extract prepared from HFF cells nor in an HeLa cell extract containing an active RNA polymerase III transcription complex (data not shown).

Sequences located on pRR8 and pUC379 are capable of competing for components of the RNA polymerase III transcription complex.

When plasmids pRR8 and pUC379 (see Figure 1) are assayed in a template exclusion experiment, significant competition for components of the RNA polymerase III complex is observed (Figure 3, lanes b and a, respectively).

In such an experiment plasmid containing HCMV DNA is incubated in HeLa cell extract under transcription conditions. Fifteen min after the addition of the extract, 0.3 µg of plasmid pArg is added and transcription is allowed to continue for another 60 min. This competitor plasmid consists of a 508 bp HindIII genomic *Drosophila melanogaster* DNA fragment carrying the gene for the major $tRNA^{Arg}$ species inserted into the HindIII site of pBR322 (30). In case the plasmid containing HCMV DNA comprises sequence elements which are recognized by components of the RNA polymerase III transcription machinery these components are sequestered onto the HCMV DNA and cannot act during transcription of the $tRNA^{Arg}$ gene, added as indicator to monitor competition (15). Depending on the stability of the complex formed, only fractions of tRNA products are synthesized compared to control experiments, in which plasmids are assayed whose DNA is not targeted by the RNA polymerase III transcription machinery.

All plasmids containing parts of the EcoRI b fragment seem to compete for components of the RNA polymerase III specific transcription complex. However competition ability increases significantly in the order of

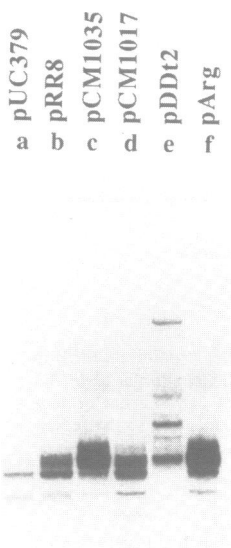

Figure 3. In vitro template exclusion experiments. 0.5µg of indicated plasmids were incubated with HeLa cell free extract and nucleotide triphospates (see MATERIALS AND METHODS). [^{3}H]UTP was present as radioactive precursor. 15 min after starting the reaction with extract, 0.3µg of plasmid pArg was added as competitor. This plasmid contains a $tRNA^{Arg}$ gene from *Drosophila melanogaster* which is efficiently transcribed in HeLa extract (see lane **f**). As positive control plasmid pDDt2 was used (lane **e**). Parts of this plasmid code for a $tRNA^{Val}$ gene from *Dictyostelium discoideum*. From this gene unusually large primary transcripts are formed in HeLa extract (Dingermann et al., 1985). None of the other tested plasmids (lanes **a** - **d**) contain genes which are transcribed in this HeLa extract. The amount of pArg transcripts varies, depending on the ability of the tested plasmids to sequester stably a component of the RNA polymerase III transcription machinery. The putative B-box like element, detected by computer search, is located on plasmids pCM1017, pRR8 and pUC379 (lanes **d**, **b** and **a**, respectively). Note that all three plasmids are able to compete to different degrees for transcription factor(s). As a negative control cosmid pCM1035 was analyzed (lane **c**). This cosmid contains a different, unrelated portion of the HCMV genome (see Figure 1).

plasmids pCM1017, pRR8 and pUC379 (Figure 3, lanes d, b and a, respectively). In fact, the part of the HCMV genome contained on pUC379 competes for components of the RNA polymerase III machinery as effective as a *bona fide* tRNA gene (Figure 3, lane e). An unrelated cosmid pCM1035, containing a different part of the HCMV genome (Figure 3, lane c) as well as pUC18 (data not shown), do not significantly interfere with transcription of the $tRNA^{Arg}$ gene. The amount of $tRNA^{Arg}$ formed *in vitro* in this latter experiment is only slightly reduced compared to that which is formed if pArg is incubated with HeLa cell extract just by itself (Figure 3, lane f). The gradual increase of competition ability observed if clones pCM1017, pRR8, and pUC379 are compared could be rationalized by the fact that the relative number of "functional" gene equivalents increases significantly in the order pCM1017, pRR8 and pUC379 due to the great differences in the absolute size of these plasmids.

Proteins specifically bind to the region containing the B-box like element.

In order to gain additional evidence for the potential biological significance of the B-box like element located on plasmid pUC379 gel retardation experiments were performed. Minute amounts (1ng) of labeled HCMV DNA (379 bp) were incubated with HeLa cell extract and analyzed on low ionic strength polyacrylamide gels. Several retarded fragments can be detected which sustain even a 5 000 fold excess of unspecific competitor DNA (Figure 4 A). This result is observed regardless whether sheared *E. coli* DNA or artificial poly(dI-dC) DNA is used as unspecific competitor DNA. Even more significant are the results if a labeled 59 bp Hinf1 subfragment instead of the total 379 bp insert of pUC379 is analyzed (Figure 4 B). Obviously major protein binding site(s) seem to be located on this 59 bp fragment and not as much on the corresponding 320 bp Hinf1 subfragment (Figure 4 C).

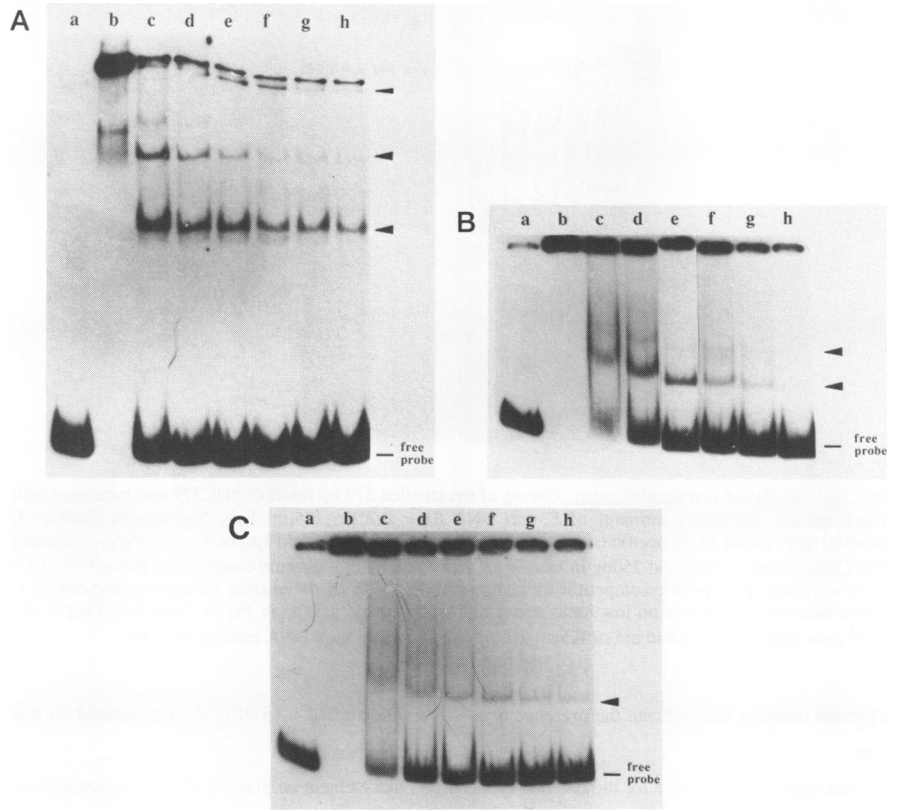

Figure 4. Gel retardation assays with the 379 bp insert of pUC379 (A) and with a 59 bp (B) and a 320 bp (C) BamH1/Hinf1 subfragment. DNAs were labeled at the BamHI sites with Klenow polymerase in the presence of [α^{32}P]dATP. The 379 bp fragment and, after digestion with HinfI, the 59 bp subfragment (B) and the 320 bp subfragment (C) were isolated by gel electrophoresis. One ng of each fragment was used for gel-shift experiments. DNA was incubated with HeLa cell extract in the presence of increasing amounts of *E. coli* DNA (0.19µg, 0.39µg, 0.67µg, 1.25µg, 2.5µg, 5.0µg in lanes **c - h**, respectively) as unspecific competitor (see MATERIALS AND METHODS). Fragments were then separated on low ionic strength polyacrylamide gels and visualized by autoradiography (12h with intensifying screen). Lane **a** contains 1ng fragment not incubated with HeLa extract, in lane **b** DNA was analyzed, which was incubated with HeLa extract without the addition of any competitor DNA. Arrowheads indicate the positions of specifically retarded DNA fragments.

This could be predicted since the 59 bp Hinf1/BamH1 fragment contains the entire putative B-box like element.

The fact that in these analyses more than one retarded fragment can be detected might be indicative for the possibility that not just one protein binds to that region. Instead, this result might present evidence that some form of a complex is sequestered, as expected for a functional transcription complex. Although the major protein binding sites seem to be located on the 59 bp Hinf1/BamH1 fragment (Figure 4 B), also the 320 bp subfragment of pUC379 seem to be able to bind a protein contained within the HeLa transcription extract (Figure 4 C). Certainly, protein binding to this fragment proves to be fairly weak. Nevertheless the formation of a

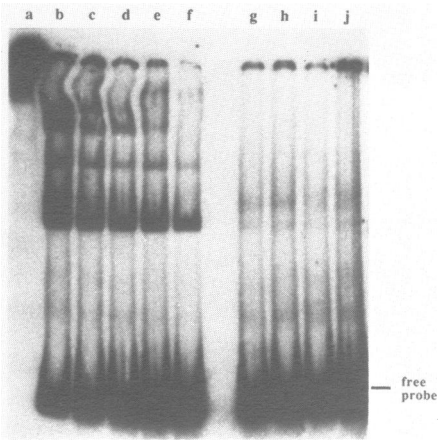

Figure 5. Gel retardation competition assay. One ng of the labelled 379 bp insert of pUC379 was incubated with HeLa cell extract. Increasing amounts of *E. coli* DNA (0μg, 0.25μg, 0.5μg, 1μg, 2μg, 4μg in lanes **a - f**, respectively) were added as unspecific competitor. Alternatively - in addition to 1μg of *E. coli* DNA - increasing amounts (33ng, 67ng, 125ng, and 250ng in lanes **g - j**, respectively) of a plasmid coding for a $tRNA^{Val}$(GUU) gene (37) were added as specific competitor for components of the RNA polymerase III transcription complex. Fragments were then separated on low ionic strength polyacrylamide gels as in Fig. 4. Note that 33ng of the $tRNA^{Val}$ gene containing plasmid are sufficient to compete away the entire DNA binding proteins.

DNA/protein complex can indicate the presence of a — yet unidentified — 5'-ICR element located on that fragment.

The most significant evidence for the function of the B box like element on plasmid pUC379 as recognition signal for components of the RNA polymerase III transcription complex is deduced from the fact that a eukaryotic tRNA gene is able to compete very efficiently for the binding proteins. In the presence of 1μg unspecific competitor DNA (compare with lane d of Figure 5) as little amounts as 33ng of a plasmid coding for a $tRNA^{Val}$(GUU) from *Dictyostelium discoideum* (37) compete away the entire binding proteins (Figure 5, lane g).

An RNA is synthesized in vivo, late after infection with HCMV from the region contained on pUC379.

Assuming the B-box like element is part of an active promoter one should be able to detect a corresponding transcription product at some stage during the infectious cycle of the virus. We decided to search for the existence of such a product by primer extension analysis. This method has lately been proven to be very reliable and extremely sensitive, especially for the detection of small RNAs (33, 34). In addition, this method offers the advantage not only to detect a transcription product but also to identify the point of transcription initiation.

A primer (primer 1) specifically recognizing the region immediately downstream of the B-box like element (see Figure 2) was used initially to prime for specific cDNA synthesis, by AMV reverse transcriptase.

RNA was prepared from mock-infected HFF cells and from HFF cells immediate early, early and late after infection with HCMV virus. Five μg of total RNA were coprecipitated with 15pmol of 5'-labeled primer fragment. After annealing, cDNA was synthesized with AMV reverse transcriptase in the presence of unlabeled

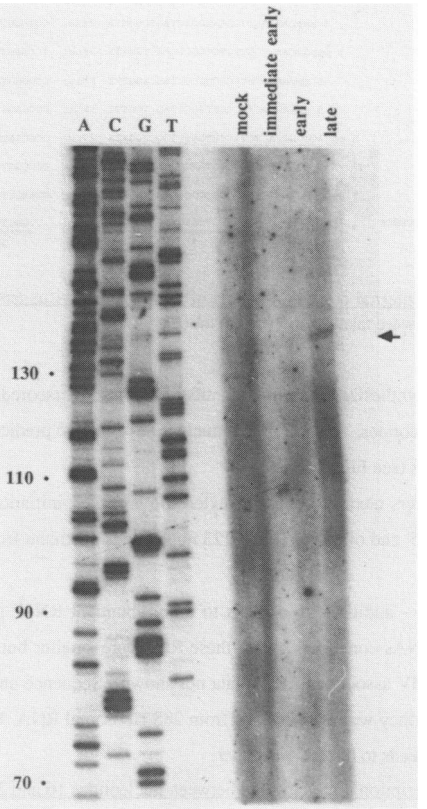

Figure 6. Primer extension analysis of whole cell RNA prepared from mock-infected cells and from cells immediately early, early and late after infection with HCMV. Five µg total cellular RNA were analyzed by primer extension. The primer (primer 1) used in this experiment anneals downstream of the B-box like element of the CMER gene (see Figure 2). As size marker served a sequence analysis according to the dideoxy termination method (34). The universal sequencing primer (pentadecamer, New England Biolabs) was phosphorylated at its 5'-end prior to annealing onto ss-DNA. This ensured identical electrophoretic mobilities of fragments generated during sequencing and in primer extension analyses.

dNTPs. cDNA products were separated on sequencing gels and visualized by autoradiography (Figure 6). RNA isolated from cells late after HCMV infection render a specific primer extension product of 135 nucleotides with primer 1 (see arrow head in Figure 6).The same primer, however, is not elongated on RNA isolated from mock infected cells, or from HFF cells isolated immediate early or early after HCMV infection.

A similar but only 123 nucleotides long cDNA fragment is observed when primer 2 (Figure 2) is annealed to HFF RNA isolated late after infection (data not shown). This latter primer is complementary to parts of the putative 3'-ICR element and anneals 12 nucleotides further upstream compared to primer 1.

These results indicate the presence of an RNA in cells late after infection with HCMV which maps to viral DNA contained on plasmid pUC379. The specificity of cDNA synthesis and the conclusion that cDNAs primed

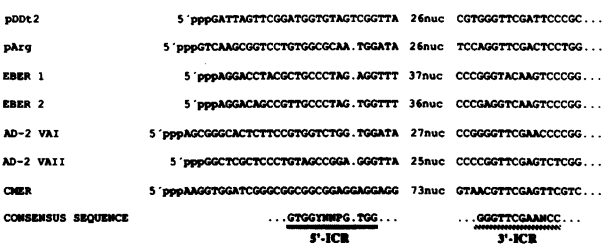

Figure 7. Comparison of gene internal control elements of tRNA genes, virus-associated genes and the CMER gene. The consensus sequences were taken from Rosa *et al.* (8).

by primer 1 and primer 2 are synthesized from the identical RNA is underscored by the fact that both cDNAs differ in size exactly by 12 nucleotides. This number of nucleotides could be predicted from the known annealing sites of the two oligonucleotides (see Figure 2).

Deduced from the size markers next to the primer extension analyses, initiation of transcription occurs 135 nucleotides upstream from the 5'-end of primer 1 and 123 nucleotides upstream from the 5'-end of primer 2 (see Figure 2).

Primer 2 - but not primer 1 - additionally anneals to more abundant RNAs present in infected as well as uninfected cells. Extended cDNAs corresponding to these RNAs are smaller but also significantly larger than cDNAs synthesized from HCMV associated RNA (data not shown). Sequence analysis of the isolated, HCMV unrelated cDNAs revealed that they were synthesized from 28S ribosomal RNA. Whether this result hints some functional evidence of CMER needs to be studied further.

The 5'-ICR of the CMER transcription unit is located between nucleotides 16 and 28.

The gene coding for CMER should not only contain a 3'-ICR but also an equivalent to the A-box or 5'-ICR, if it indeed is transcribed by RNA polymerase III. Such an element could be identified after the transcription initiation point had been determined. As depicted in Figure 7, this element shows extremely good similarity to corresponding elements in tRNA genes or in virus-associated class III genes (8). The relatively close position of the element to the point of transcription initiation is typical. On the other hand, the distance between 5'- and 3'-ICR is fairly large. This as well as the lack of a highly conserved T residue at position 2 of the consensus sequence may be the reason why the element had not been detected earlier.

DISCUSSION

In the present study we have detected a transcription unit located on the EcoRI b fragment of the common physical map of the HCMV (strain AD 169) genome. This unit is most likely to be transcribed by RNA polymerase III based on functional analyses. This presumption is supported by the observation that the gene contains gene internal regions, highly homologous to the typical non-contiguous promoter elements of eukaryotic class III genes. This unit is significantly capable of competing for factors specific for RNA polymerase III directed transcription. This could be demonstrated by preincubation assays, where the ability of a

Figure 8. Partial sequence complementarity between CMER and human 28S rRNA.

given template is tested to sequester RNA polymerase III specific transcription factors. These assays are performed by programming a transcription reaction with DNA I (here HCMV DNA) at time 0. After the predetermined time (15 min in these studies), DNA II (a *bona fide* $tRNA^{Arg}$ gene) is added and transcription is allowed to proceed for another 60 min. The quantitation of DNA II transcription under these conditions is a measure of the ability of DNA I to bind transcription factors. The premise of this assay rests on the demonstration that the transcription factors act stoichiometrically and are not recycled from a template once the stable transcription complex has been formed. These assays have previously been interpreted as a means to assay the ability of a tRNA gene or of the VA RNA genes to sequester transcription factors TFIIIB and TFIIIC (39).

In case of the CMER gene competition for component(s) of the RNA polymerase III complex increases drastically, inversely proportional to the "unspecific" viral DNA contained on the tested plasmid. This may be caused by alternative reasons. The increase in competitiveness could be explained by the relative increase in number of specific templates if equal amounts of smaller plasmids compared to cosmids are assayed. Alternatively or in addition, other proteins present in the HeLa transcription extract may be assembled onto a larger region of the viral genome, which thereby might interfere with the binding of a RNA polymerase III specific protein. Such a dual function of a genomic region coding for virus associated RNAs has been described for *H. saimiri* (4).

Gel retardation experiments support the results obtained in template exclusion assays since proteins — most likely components of the RNA polymerase III transcription complex — can stably associate with this region of the viral genome. We were unable, however, to demonstrate transcriptional activity of the gene *in vitro*, neither with HeLa extract nor with extract prepared from HFF cells. A specific transcription product, however, could be detected by primer extension analyses of total RNA isolated from HFF cells late after infection with HCMV.

Many tRNA genes from different organisms are known to be inactive *in vitro*. Although such genes contain *bona fide* gene internal promoter elements it could be demonstrated that 5'-flanking regions exert drastic effects on template activity of certain tRNA genes (39). Therefore the inactivity of the CMER gene in *in vitro* transcription assays is not too much of a drawback. However, due to this *in vitro* inactivity it was not possible to test, whether CMER synthesis might be sensitive or resistant to α-amanitin. Such an experiment would additionally argue in favor or against the involvement of RNA polymerase III in CMER synthesis. Further indication for RNA polymerase III directed synthesis of the CMER would be the demonstration of the absence of a poly-A tail and of a cap-structure on CMER. However, presently it is difficult to conduct such experiments since the amount of detectable RNA in infected HFF cells is fairly low.

Indeed the low amounts of CMER detectable in infected cells is rather unusual if compared to adenovirus where VA RNAs are synthesized in high amounts during lytic replication. However in case of other herpesviruses (EBV and *H. saimiri*) small RNAs are only detected in persistently infected lymphoid cells. Epithelial cells, productively infected by *H. saimiri* do not contain significant amounts of small RNAs (4). Instead, the coding region for these RNAs obviously overlaps with the coding region for a number of polyA$^+$ RNAs which are synthesized in permissively-infected monolayer cells *in vitro* (4). Thus the restricted expression of CMER in lytically infected cells may not be too surprising in view of other viral systems. Probably the drastic decrease of competitiveness in template exclusion assays of different clones containing all the CMER coding region but different amounts of CMER-unrelated HCMV DNA may indicate an additional function of this part of the genome similar to the corresponding region in *H. saimiri*. Satisfactory cell culture systems to study persistence of HCMV are not available to date.

Alternatively, the low amount of detectable CMER may be caused by the presence of an inefficient promoter. Although sequence similarities of the 5'-ICR with established consensus sequences are very significant, the T to C transition at the 5'-end of the 5'-ICR is unique to our knowledge. Whether this reflects a micro-heterogeneity within the particular viral isolate studied or whether this is typical for HCMV needs to be determined further. Also the relative high number of nucleotides located between both control regions may be responsible for reduced promoter strength. The distance between ICRs, however, can vary indeed to a great extent between different class III genes, i.e. in tRNA genes coding for large extra arms or intervening sequences.

Nevertheless the low abundance of CMER late after infection does not necessarily argue against a specific function of this RNA. However, so far we have no clues what the function of this RNA could be as there is little known in general about the function of virus associated RNAs. There are indications that small RNAs encoded by *H. saimiri* might be important for the regulation of T-cell growth (4). VAI RNA has been proven to prevent phosphorylation of eIF-2 (40, 41), and other than the fact that EBER RNAs can functionally substitute VAI RNA (42) and that EBER RNAs exhibit some complementarity to 18S rRNA (7), the physiological role of these RNAs is unknown.

The probably fortuitously obtained result that primer 2 picks up 28S rRNA (data not shown) and a previous report that the EcoRI b fragment hybridizes to the 5'-portion of the 28S rRNA gene (36) prompted us to compare more carefully CMER with ribosomal RNAs. There is indeed significant complementarity between parts of CMER and human 28S rRNA (Figure 8).

Whether CMER might be another example for the involvement of a virus associated RNA in translation processes of infected cells — as it is the case for VA RNAs and probably also for EBER RNAs — is not known, yet. Studies evaluating the functional significance of HCMV/ 28S rRNA-complementarity are currently in progress.

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*To whom correspondence should be addressed

+Present address: University of North Carolina, Department of Biology, Charlotte, NC 28223, USA

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