The DNA sequence coding for the 5’ untranslated region of herpes simplex virus type 1 ICP22 mRNA mediates a high level of gene expression

Anna Greco, Denis Simonin, Jean-Jacques Diaz, Laure Barjhoux, Karine Kindbeiter, Jean-Jacques Madjar* and Thierry Massé

Immuno-Virologie Moléculaire et Cellulaire, CNRS UMR30, Faculté de Médecine, Rue Guillaume Paradin, 69372 Lyon CEDEX 08, France

The sequence coding for the 5’ untranslated region (UTR) of ICP22 mRNA of herpes simplex virus type 1 has been tested for its ability to regulate gene expression. This sequence was placed in frame with the chloramphenicol acetyltransferase (CAT) coding sequence and under the control of the simian virus 40 early promoter–enhancer. Under these conditions, the sequence coding for the 5’UTR led to an increase of about 13-fold in CAT activity, measured during transient expression. The use of mutants with progressive deletions within the sequence coding for the 5’UTR allowed localization of the sequence responsible for the enhancement of gene expression to the first exon of the ICP22 gene. Precise quantification of hybrid ICP22-CAT mRNA showed that the sequence coding for the 5’UTR induced an increase in the amounts of transcripts, which resulted in a parallel increase in CAT activity. This increase in the level of hybrid ICP22–CAT mRNA is not the result of an increase in mRNA stability, nor is it due to more efficient nucleo-cytoplasmic transport of the transcripts. Moreover, the distribution of hybrid mRNA in the different ribosomal populations indicates that the 5’UTR of ICP22 mRNA does not induce a preferential recruitment of the transcripts by the translational apparatus. Taken together, these results indicate that a cis-acting element located in the sequence coding for the 5’UTR of ICP22 mRNA can mediate a high level of gene expression independently of the viral promoter and of viral trans-acting factors.

Introduction

The herpes simplex virus type 1 (HSV-1) genome encodes at least 70 genes whose expression is temporally regulated in a cascade fashion. These genes are designated immediate early or α, early or β, leaky late or γ1 and true late or γ2 (Honess & Roizman, 1974). The five α genes (ICP0, ICP4, ICP22, ICP27 and ICP47) are transcribed in the absence of de novo viral protein synthesis, whereas expression of β, γ1 and γ2 genes requires α proteins. Expression of α proteins and regulation by viral and cellular factors is thus a key event in initiating the viral cycle (Garcin et al., 1990; Massé et al., 1990a; Roizman & Sears, 1993). Immediately after HSV-1 infection, host cell protein synthesis is selectively inhibited while viral transcripts are efficiently translated (Fenwick & Walker, 1978; Honess & Roizman, 1974). This differential expression of cellular and viral genes occurs at transcriptional and post-transcriptional levels, while both cellular and viral mRNAs are destabilized by at least one virion-associated factor (Jones & Roizman, 1979; Kwong & Frenkel, 1989; Oroskar & Read, 1989; Weinheimer & McKnight, 1987). Immediate early gene expression is, in part, regulated by an abundant structural component of the virion, VP16, which is delivered into cells upon infection (Batterson & Roizman, 1983; O’Hare & Hayward, 1985). However, preferential expression of the viral α genes may also be due to the presence of structural features in their sequence. In particular, the sequence coding for the untranslated regions may increase transcription, mRNA stability or translation efficiency (Blair et al., 1987; Garfinkel & Katze, 1992; Geballe & Gray, 1992; Guzowski & Wagner, 1993; Huang et al., 1993; Huang & Schneider, 1991; Leong et al., 1990; Lindquist & Petersen, 1990; Meervitch et al., 1991; Pederson et al., 1992; Tedder et al., 1989; Weir & Narayanan, 1990). Sequences located downstream of the transcription start site may be the target for trans-acting factors, allowing regulation of gene expression at transcriptional and/or post-transcriptional levels.

Although the promoter activity of many HSV-1 genes has been rather well characterized, only the role of the DNA sequence coding for the 5’ untranslated region
(5' UTR) of some mRNAs of β, γ1 and γ2 genes has been studied. Full expression of some β, γ1 and γ2 genes requires elements within the sequences coding for the 5' UTR (Blair et al., 1987; Coen et al., 1986; Guzikowski & Wagner, 1993; Huang et al., 1993; Pederson et al., 1992; Romanelli et al., 1992; Tedder et al., 1989; Weir & Narayanan, 1990). The sequences coding for the 5' UTR of HSV-1 α mRNA range in length from 148 to 530 nucleotides. Among the mRNAs coding for the five α products, those of ICP22 and ICP47 have a very similar 5' UTR (Rixon & Clements, 1982). These two 5' UTRs are mainly encoded by the short repeat regions, have a high GC content and a single intron (Fig. 1b). Furthermore, a strong homology exists between the beginning of the 5' UTR of ICP22 and ICP47 mRNA and that of ICP4 (A. Greco, D. Simonin, J.-J. Diaz, L. Barjhoux, K. Kindbeiter, J.-J. Madjar & T. Massé, unpublished results).

A strategy has been designed to investigate the role of the sequence coding for the 5' UTR of ICP22 mRNA on gene expression, independently of the viral promoter and viral trans-acting factors. A construct was made of the complete sequence coding for the 5' UTR of ICP22, including the intron sequence and the initiation codon together with the surrounding nucleotides, and of the Escherichia coli chloramphenicol acetyltransferase (CAT) coding sequence. A set of deletion mutants in the sequence coding for the 5' UTR were derived from this initial construct. The enzymatic CAT activity was measured after transient expression of each of these chimeric genes. Measurement of CAT activity and quantification of mRNA revealed that the sequence coding for the 5' UTR contains elements that induce an increase in the amount of the hybrid ICP22–CAT mRNA. This increase is strongly correlated with the increase observed in the CAT activity. Analyses of half-lives and polyribosomal distribution of hybrid CAT mRNA indicate that the 5' UTR does not stabilize the transcripts and does not induce a preferential recruitment of the ICP22–CAT hybrid mRNA in the polyribosomal fractions.

### Methods

**Cells.** HeLa cells and human epidermoid carcinoma (HEp2) cells were grown as monolayers in Eagle's minimum essential medium supplemented with 10% heat-inactivated newborn calf serum.

**Plasmids.** Construction, isolation and preparation of plasmids were carried out using standard procedures (Ausubel et al., 1987). Plasmid pCMVβ is composed of the E. coli β-galactosidase gene under the control of the cytomegalovirus (CMV) immediate early promoter–enhancer (Clontech). The pSV2CAT plasmid is composed of the simian virus (SV40) early promoter–enhancer followed by 70 bp from the sequence downstream of the SV40 transcriptional start site, cloned into the plasmid upstream of the sequence coding for the bacterial CAT mRNA (Gorman et al., 1982).

Plasmid pSV22-1 contains the sequence coding for the 5' UTR of HSV-1 ICP22 mRNA cloned between the SV40 early promoter–enhancer and the CAT coding sequence. In order to generate pSV22-1, a new cloning vector derived from pSV2CAT was first constructed by mutagenesis in phage M13 (Fig. 1c). The CAT initiation codon and its surrounding region were replaced by a polylinker sequence, allowing the cloning of any sequence including the initiation codon in frame with the remaining CAT coding sequence. A 529 bp Narl–FokI fragment (from position +5 to position +533 of the ICP22 gene), including the translation initiation codon at position +518 was inserted in frame with the CAT coding sequence. This fragment comprises the sequence coding for most of the 5' UTR, together with the first 16 bases of the open reading frame of the ICP22 gene. Plasmids pSV22-2 to pSV22-6 were derived from pSV22-1 by successive deletions as shown in Fig. 1(d). In plasmid pSV22-1c, the 227 bp Smal–EcoNI fragment was replaced by the 59 bp Smal–EcoNI fragment lacking the sequence coding for the intron. This smaller fragment was obtained after reverse transcription of the mRNA transcribed from pSV22-1, followed by PCR amplification of the cDNA. PCR was carried out with the antisense primer B (5' GGAGAATAGGCCACGTTTTACCGCATA 3'), located in the CAT coding sequence, and the sense primer E (5' GGGACCTAGGCTTTTGC 3'), proximal to the transcriptional cap site (see Fig. 1b for location and 5' to 3' orientation of the primers). After digestion of the PCR product with Smal and EcoNI, the 59 bp fragment was selected and inserted between the Smal–EcoNI restriction sites of pSV22-1.

Plasmid pSV22-1AEco was derived from pSV22-1. The plasmid was digested with EcoRI at the unique restriction site in the CAT coding sequence, blunt-ended by digestion with T4 DNA polymerase and religated (Greco et al., 1993).

**Transfection assays, RNA purification and measurement of CAT activity.** Cells were transfected by the calcium phosphate precipitation procedure (Sambrook et al., 1989). Briefly, 1.2 x 10⁶ cells were plated onto 100 mm diameter Petri dishes and incubated at 37 °C for 16 to 17 h. At 1 h before transfection, the medium was replaced by 10 ml of fresh medium. Then 0.5 ml of a transfection mixture containing 15 μg of total DNA (7 μg of the relevant CAT plasmid, 3 μg of pCMVβ and 5 μg of pUC18) in 1 x PBS buffer (140 mm-NaCl, 5 mm-KCl, 0.75 mm-NaH₂PO₄, 2H₂O, 6 mm-glucose and 25 mm-HEPES, pH 7.0) was added to the medium. Twenty-four hours after transfection the medium containing the transfection precipitate was removed. Cells were scraped off and RNA was purified immediately from 90% of the cells. The remaining 10% of cells were used for protein extraction. pCMVβ was used as an internal control for determination of the transfection efficiency. Each transfection was performed in duplicate and all the experiments were repeated at least three times.

Total cytoplasmic and/or nuclear RNA was extracted after cell lysis with Nonidet P-40 at a final concentration of 0.1%, followed by proteinase K digestion and phenol–chloroform extraction (Ausubel et al., 1987). Contaminating DNA was removed by digestion with 10 units of DNase I (RNase-free) per assay.

Protein extracts were prepared essentially as previously described (Gorman et al., 1982). However, instead of sonication, cell lysis was achieved by treatment with Nonidet P-40 at a final concentration of 0.7%. Each CAT assay used 1% of the protein extract. CAT activities were measured by following the kinetics of chloramphenicol acetylation and by determining the initial rate of the enzymatic reaction for each assay in conditions where the substrate was not limiting. The initial velocity of the reaction was then converted into pmol of chloramphenicol acetylated per min as previously described (Desbois et al., 1992). Only the data obtained from experiments where the transfection efficiencies were of the same order of magnitude after measurement of the β-galactosidase activities (Ausubel et al., 1987) were taken into
Expression of hybrid HSV-1 ICP22 5'UTR–CAT mRNA

A 529 bp fragment (thick line) containing the sequence coding for the first 16 bases of the ICP22 coding sequence was inserted between location and 5' to 3' orientation of the immediate early mRNA repeats, and TR s and IR s for terminal and internal short repeats. The species are indicated by arrows and position of introns are also shown. Shown as continuous lines. Major repeated sequences are represented of the HSV-1 genome (McGeoch et al., 1988).

Mutagenesis in M13

(a) SV40 early promoter–enhancer +1 ATG SfiI :HindIII

CAT oligonucleotide

HindIII SmaI XhoI

Mutagenesis in M13

(b) Primer E ~ A2

0 5 10 15 115 120 125 130 135 140 145 150 kbp

TRIL U5 -1 ICPO ICp4 ICp7

ICP0 ICp4 ICP47

SfiI HindIII NarI NruI Sinai EcoNI XhoI EcoRI

ICP0 ICp4 ICP47

XhoI

ICP22

pSV22-1

pSV22-2

pSV22-4

pSV22-5

pSV22-6

pSV22-1c

Primer

S E C A2 B

Fig. 1. Construction of the series of pSV22 plasmids. (a) Mutagenesis in phage M13. The ATG initiation codon and the surrounding region of the CAT gene from pSV2CAT were changed for a polylinker sequence resulting in a new cloning vector. (b) Cloning of the sequence coding for the 5'UTR of ICP22 mRNA. The upper part shows a map of the HSV-1 genome (McGeoch et al., 1985, 1986, 1988; Perry & McGeoch, 1988). Long and short unique sequences (U, and U, s) are shown as continuous lines. Major repeated sequences are represented by open boxes labelled TR, and IR, for terminal and internal long repeats, and TR, and IR, for terminal and internal short repeats. The location and 5' to 3' orientation of the immediate early mRNA species are indicated by arrows and position of introns are also shown. A 529 bp fragment (thick line) containing the sequence coding for the 5'UTR of the ICP22 mRNA including the ATG initiation codon and the first 16 bases of the ICP22 coding sequence was inserted between the Xmal and XhoI sites of the new cloning vector (a) giving rise to account. All the CAT activities were normalized by the correcting factors derived from the ß-galactosidase activities.

S1 nuclease mapping and reverse transcription–PCR. The initiation site of pSV22-1 transcription was determined by S1 nuclease mapping using a 50 base-long synthetic oligonucleotide, which was 5' end-labelled with [32P] (5' GCCCTTCCTACACTGGAATATAGATCA-GAGGCCGAGGCGGCTTCGGC 3'). This probe, called oligonucleotide S, corresponds to the sequence spanning the transcription start site of the SV40 early gene (Fig. 1b). Between 10 and 20 µg of total RNA was used in these S1 mapping experiments, which were performed as described previously (Ausubel et al., 1987). The size of the S1-protected fragments was measured after electrophoresis in a 8% (w/v) polyacrylamide gel containing 8:3 M-urea.

Correct splicing of the hybrid CAT mRNA was verified by reverse transcription followed by amplification by PCR. First, CAT cDNA was synthesized at 37 °C for 1 h from 2 µg of total cytoplasmic RNA, starting from the antisense oligonucleotide primer B complementary to the CAT coding sequence. A part of the reverse transcription product was then amplified by PCR in which oligonucleotides B and C were used as primers. Oligonucleotide C (5' ACGACAGAAA-CCCACGGGT C 3') is a sense primer identical to the sequence located immediately upstream of the intron of the ICP22 primary transcript (Fig. 1b). The 50 µl PCR reaction mixture, containing 5% of the reverse transcription products in PCR buffer (25 mM-Tris–HCl pH 8.3, 20 mM-KCl, 2 mM-MgCl2, 0.1 µg/µl BSA, 120 µM-dNTP, 1 µM of each primer and 1 unit of Appligene Taq polymerase) was first denatured at 92 °C for 3 min. It was then amplified for 40 cycles (92 °C for 42 s, 55 °C for 60 s and 72 °C for 90 s) and cooled at 10 °C for 10 min. An aliquot corresponding to 20% of the PCR products was analysed by electrophoresis in a 1% agarose gel with 0.5 µg/ml ethidium bromide (Mullis & Faloona, 1987).

cDNA quantification by competitive PCR. After transfection, CAT cDNA was synthesized by reverse transcription from the total cytoplasmic RNA of about 20000 transfected HeLa cells. For each given quantification, an identical amount of about 5 to 10% of the CAT cDNA was co-amplified by PCR with a competitive double-stranded pSV22-1AEco DNA template, together with 1 µM each of antisense oligonucleotide B and sense oligonucleotide A2 (5' CGTGGATATATCCCAATGGC 3'). These are both located at an equal distance from a unique EcoRI site in the CAT coding sequence (Fig. 1b). For each CAT cDNA, this co-amplification was carried out five times with the competitor DNA at successive dilutions. After an amplification of 40 cycles, the PCR products from the cDNA and from the competitor DNA were discriminated by electrophoretic separation of the resulting fragments in a 1:2% agarose gel. Only the DNA amplified from cDNA to be quantified was therefore comigrated in the gel. After ethidium bromide staining, comparison of the intensity of the resulting fragments in a competition series allowed the quantification of the amount of CAT cDNA. Experimental details for quantifying the CAT cDNA have been published elsewhere (Greco et al., 1993).

Determination of hybrid CAT mRNA half-lives. HeLa cells were transfected with the relevant hybrid ICP22–CAT plasmids. After 20 h pSV22-1. The positions of some restriction enzymes relative to the ICP22 gene are indicated. The transcription start site is indicated by a bent arrow. The location of the intron sequence between the Smal and EcoNi sites in the sequence coding for the 5'UTR of ICP22 mRNA is indicated by a dotted line. Deletions are indicated by hatched boxes. The locations of the different oligonucleotides used here are indicated below; arrows specify the 5' to 3' orientation of the molecules.
the medium was replaced and 2 h later cells were incubated with fresh medium containing 5 μg/ml of actinomycin D. Total cytoplasmic RNA was extracted at 1 h intervals until 8 h after addition of actinomycin D. CAT mRNA decay was estimated after Northern blotting. A 1600 bp HindIII–BamHI DNA fragment purified from pSV2CAT containing the entire CAT coding sequence was used as a probe and was 32P-labelled by random priming. Results were normalized to the amount of ribosomal protein S14 mRNA detected by hybridization with a probe corresponding to the entire S14 coding sequence from plasmid pCS14-12 (Rhoads et al., 1986). After autoradiography of the blot, bands containing CAT mRNA were cut out from the filters and the radioactivity was measured by scintillation counting.

Sucrose gradient fractionation of polyribosomes. This was carried out with post-mitochondrial supernatants from transfected HeLa cells as previously described (Diaz et al., 1993). Briefly, transfected cells were washed in PBS, scraped off from the monolayer and collected in PBS. After centrifugation, the cells were resuspended in ice-cold buffer (250 mm sucrose, 50 mm-Tris–HCl pH 7.4, 25 mm-KCl and 5 mm-MgCl2) and lysed by addition of Triton X-100 to a final concentration of 0.5%. Nuclei and mitochondria were pelleted by two successive centrifugations at 750 g and 12000 g. The post-mitochondrial supernatant was layered onto a 10 to 40 % linear sucrose gradient made in the same buffer but containing 2mM-DTE. After separation by ultracentrifugation in a Beckman SW41Ti rotor at 40000 r.p.m, for 150 min at 4 °C, fractions were collected from the top of the gradient. Total RNA was purified from fractions below a coefficient of sedimentation of 40S and from other fractions containing individual ribosomal subunits, ribosomes and polyribosomes. Quantification of CAT cDNA synthesized from the CAT mRNA contained in each fraction was performed by competitive PCR as described above.

Results

Validity of the experimental strategy

For investigating the ability of the sequence coding for the 5′UTR of the ICP22 mRNA to regulate gene expression, plasmid pSV22-1 was first derived from pSV2CAT. It comprises the complete SV40 early promoter–enhancer, followed by 70 bp of the downstream SV40 sequence (relative to the start site) and 513 bp of the sequence coding for the 5′UTR of ICP22 mRNA; the following 16 bp were cloned in frame with the 5′UTR of SV40 early mRNA. Similarly, pSV22-4, pSV22-5 and pSV22-6 were derived from pSV22-1 by removal of the SfiI–NruI, SfiI–SmaI and SfiI–EcoNI fragments respectively. pSV22-1c lacks only the sequence coding for the intron (from positions +247 to +414 relative to the ICP22 gene). The influence of gene expression of the sequence coding for the 5′UTR of ICP22 mRNA and of deletions within this sequence was studied by assaying CAT activity of protein extracts from HeLa cells after transfection with the different CAT plasmids.

To evaluate the transfection efficiency, CAT plasmids were transfected together with pCMVβ in conditions where their respective expression levels were still directly proportional to their concentrations. In addition, cells were recovered 24 h post-transfection because CAT and β-galactosidase activities were found to increase linearly for at least 30 h and because the ratio of CAT activities obtained for the different mutants remained constant during this time (data not shown). Under our experimental conditions, 100 % of the cells expressed β-galactosidase, albeit to different extents, as visualized after an in situ β-galactosidase assay.

We verified that the hybrid ICP22–CAT mRNA transcribed from pSV22-1 was initiated correctly. S1 nuclease mapping experiments using the antisense SV40 oligonucleotide S as a probe showed that mRNA transcribed from pSV2CAT and from pSV22-1 had the same transcription initiation sites (Fig. 2a). Three major bands were visible and corresponded to protected fragments of different sizes. They are derived from transcripts initiated at the various transcription start sites of the SV40 early gene. Identical results were obtained with a 32P-labelled antisense RNA synthesized in vitro and used as a probe in RNase protection assays (data not shown). These results indicate that the sequence

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**Fig. 2.** S1 nuclease mapping and reverse transcription–PCR analyses for determination of the correct transcriptional initiation sites, nuclearcytoplasmic distribution and splicing. (a) S1 mapping analysis of total cytoplasmic (i) and nuclear (ii) RNA extracted from HeLa cells transfected with pSV2CAT (lanes 1) or pSV22-1 (lanes 2). The SV40 antisense oligonucleotide S was used as a 32P-labelled probe. (b) Splicing of hybrid ICP22–CAT primary transcript. DNA from pSV2CAT and pSV22-1, as well as cDNA synthesized from total cytoplasmic RNA extracted from HeLa cells transfected with different plasmids, were amplified by PCR. After separation by agarose gel electrophoresis the fragments were stained with ethidium bromide. The control amplification, without a template, is in the first lane. The size of markers in bp is indicated to the left. The positions of the PCR products corresponding to the unspliced (726 bp) and the spliced (558 bp) templates are indicated to the right.
Expression of hybrid HSV-1 ICP22 5’UTR–CAT mRNA

To verify whether the 168 nucleotide intron present in the 5’UTR of the ICP22 primary transcript was correctly spliced, the hybrid CAT mRNAs transcribed from the different plasmids were analysed by reverse transcription and PCR using primer B for reverse transcription and primers B and C for amplification. Primer B is located in the CAT coding sequence and primer C is located immediately upstream of the sequence coding for the intron. The results are shown in Fig. 2 (b). A 558 bp fragment was obtained by amplification from pSV22-1 to pSV22-5 cDNAs, which corresponded to the expected length of the correctly spliced hybrid ICP22-CAT mRNA. As anticipated, no amplification was obtained from either pSV2CAT DNA, pSV2CAT cDNA or pSV22-6 cDNA, which should not hybridize to primer C. A 726 bp fragment was amplified from pSV22-1 DNA, which was used as a control template and contained the sequence coding for the intron. Similar results were obtained in S1 mapping experiments using a 32P-labelled riboprobe in the opposite sense to the RNA (data not shown). Taken together, these results show that the intron is correctly spliced from the primary transcript of each plasmid containing the sequence encoding it.

Stimulation of gene expression

To determine the involvement of the sequence coding for the 5’UTR of ICP22 in regulation of gene expression, plasmid pSV22-1 was assayed in transient expression experiments. CAT activities were measured after transfection of HeLa cells with either pSV22-1 or pSV22CAT. Results obtained from four independent experiments are shown in Table 1. CAT activities measured after transfection of cells with pSV22-1 are approximately 10- to 20-fold higher than the CAT activities obtained after transfection of cells with pSV2CAT. Therefore the sequence coding for the 5’UTR of ICP22 mRNA leads to an increase in CAT expression when cloned upstream of the CAT coding sequence. Other plasmids derived from pSV22-1 were obtained by progressive deletions from the upstream side of the sequence coding for the 5’UTR and an additional construct was obtained by removal of the sequence coding for the intron. No significant change in the CAT activity was observed when the downstream SV40 region or the 90 bp NarI–NruI fragment of the ICP22 gene were deleted. However, deletion of the 100 bp NruI–SmaI fragment in the sequence coding for the 5’UTR of ICP22 mRNA led to an 80% decrease in CAT activity. Moreover, removal of the 225 bp SmaI–EcoNI fragment induced an additional decrease in CAT activity. With the construct deprived of the sequence coding for the intron (pSV22-1c), CAT activity appears slightly lower than that obtained with pSV22-1 to pSV22-4 (Table 1). Similar results were obtained in experiments carried out with HEp-2 cells instead of HeLa cells (data not shown). All these results show that the sequence coding for the 5’UTR of ICP22 mRNA stimulates CAT expression. Moreover, sequences within the NruI–EcoNI fragment contain elements required for efficient expression, since their deletion abolishes the increase in CAT activity. We therefore concluded from these experiments that the sequence coding for the 5’UTR of ICP22 mRNA is contributing to the regulation of hybrid ICP22–CAT gene expression at either the transcriptional or post-transcriptional level, or both.

Increase in the amount of hybrid CAT mRNA

To investigate whether the sequence coding for the 5’UTR of ICP22 mRNA was involved in regulating the level of their transcripts, we measured the amount of the different hybrid CAT mRNA transcribed from the various plasmids containing the CAT gene. RNA was purified from a fraction of the cells used to measure the CAT activities. CAT cDNA was synthesized by reverse transcription from total cytoplasmic RNA and quantified by competitive PCR, using oligonucleotides A2 and B which are both located at an equal distance from a unique EcoRI site in the CAT coding sequence. Their amounts reflect the amount of CAT mRNA because reverse transcription and competitive PCR were carried out in non-limiting conditions (Gilliland et al., 1990). Results presented in Fig. 3 show that the amount of CAT cDNA synthesized from mRNA transcribed from pSV22-1 is higher than the amount from pSV2CAT. About 515 to 1070 molecules of CAT cDNA were synthesized from mRNA transcribed per cell transfected with pSV22-1, whereas about 60 to 170 molecules of CAT cDNA were synthesized from mRNA transcribed

Table 1. CAT activity in transfected cells

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Average CAT activity*</th>
<th>%†</th>
<th>Stimulation§</th>
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<tr>
<td>pSV2CAT</td>
<td>3.8 ± 0.9</td>
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<tr>
<td>pSV22-1</td>
<td>44.4 ± 7.8</td>
<td>85.6 ± 5.9</td>
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</tr>
<tr>
<td>pSV22-2</td>
<td>50.5 ± 8.3</td>
<td>97.7 ± 3.9</td>
<td>13</td>
</tr>
<tr>
<td>pSV22-4</td>
<td>42.5 ± 13.7</td>
<td>80.8 ± 18.4</td>
<td>11</td>
</tr>
<tr>
<td>pSV22-5</td>
<td>8.6 ± 2.4</td>
<td>163 ± 3.1</td>
<td>2</td>
</tr>
<tr>
<td>pSV22-6</td>
<td>22 ± 0.6</td>
<td>43 ± 0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>pSV22-1c</td>
<td>28.9 ± 10.3</td>
<td>557 ± 19.9</td>
<td>7</td>
</tr>
</tbody>
</table>

* Calculated from four different experiments.
† Expressed in pmol of chloramphenicol acetylated per min of enzymatic reaction.
‡ Calculated from four different experiments.
§ Calculated by comparison to the activity obtained with the control pSV2CAT.
cDNAs were not significantly different when fragments increase in the amounts of CAT transcripts. The digested with corresponding to lanes I to 7 respectively. The PCR products were with the following amounts of the pSV22-1AEco competitor DNA: of the CAT coding sequence. The amounts of CAT for the 5'UTR of ICP22 mRNA was inserted upstream experiments, the results of which are shown in Table 1. CAT eDNA was synthesized from total cytoplasmic RNA purified from HeLa cells transferred with the indicated plasmids. In each series, an identical amount of the reverse transcription product was co-amplified by PCR from total cytoplasmic RNA extracted from one transfected cell. RNA was extracted from cells used in one of the four experiments, the results of which are given in Table 1. Results are expressed as the average number of CAT cDNA molecules synthesized per cell transfected with pSV2CAT. Therefore it can be assumed that the amount of CAT cDNA molecules increases from five- to 15-fold when the sequence coding for the 5'UTR of ICP22 mRNA was inserted upstream of the CAT coding sequence. The amounts of CAT cDNAs were not significantly different when fragments SfiI–HindIII or HindIII–NruI were removed (Fig. 3b). In contrast, the amount of CAT cDNA decreased dramatically when fragment NruI–SmaI and fragment SmaI–EcoNI were deleted. Here again, similar results were obtained in experiments carried out with HEp2 cells (data not shown). These data indicate that the sequence coding for the 5'UTR of ICP22 mRNA cloned upstream of the CAT coding sequence leads to a substantial increase in the amounts of CAT transcripts. The responsive sequences are therefore located between NruI and EcoNI. Moreover, comparison of results obtained from pSV22-1 and from the corresponding pSV22-1c deletion mutant without the sequence coding for the intron shows that both types of CAT mRNA accumulate at a similar level. This suggests that the intron present within the 5'UTR of the ICP22 primary transcript has little if any significant effect on the amount of CAT mRNA. Comparison of results given in Table 1 and in Fig. 3(b) show that the level of CAT activity is proportional to the amount of CAT cDNA made from cytoplasmic mRNA.

**No stabilization and no preferential nucleo-cytoplasmic transport of hybrid CAT mRNA**

To test whether the difference in the level of hybrid CAT mRNA could be caused by changes in mRNA stability, the half-lives of the different mRNA species were measured after actinomycin D treatment. Cells were transfected with either pSV2CAT, pSV22-1 or pSV22-6, the latter two containing the entire and the smallest sequence coding for the 5'UTR of ICP22 mRNA respectively. The relative amount of cytoplasmic CAT mRNA extracted at various times after addition of actinomycin D was estimated by Northern blot analysis. The results shown in Fig. 4 indicate that the different CAT mRNAs have very similar half-lives of about 3-5 h. The results indicate that the 5'UTR of ICP22 mRNA does not stabilize the transcripts, because mRNAs transcribed from the different CAT expression plasmids were degraded at about the same rate, whether or not they contained the 5'UTR of ICP22 mRNA.

In addition, to test whether the difference in the level of cytoplasmic mRNA was the consequence of more efficient nucleo-cytoplasmic transportation, the relative amounts of the CAT mRNA transcribed from pSV2CAT and from pSV22-1 were compared in the nucleus and in the cytoplasmic fractions. Total nuclear and cytoplasmic RNAs of the corresponding cells were submitted to S1 nuclease mapping using the antisense SV40 probe oligonucleotide S which was 32P-labelled. Comparison of intensities of the protected fragments from nuclear and cytoplasmic mRNA did not show significant differences in the relative nucleo-cytoplasmic distribution of CAT mRNA transcribed from pSV2CAT and from pSV22-1 (Fig. 2a). This indicates that CAT mRNAs are transported to the cytoplasm with the same efficiency, whether or not they contain the 5'UTR of ICP22 mRNA. Therefore the observed increase in the amount of hybrid CAT mRNA cannot be the result of an increase in the stability of the corresponding transcripts. Nor is it due to a more efficient nucleo-cytoplasmic transport of the ICP22-CAT mRNA.
Expression of hybrid HSV-1 ICP22 5'UTR-CAT mRNA

Fig. 4. Determination of CAT mRNA half-lives from Northern blot analyses. (a) Autoradiograms of Northern blots. HeLa cells were transfected with three different plasmids, and incubated in the absence (-) or presence (+) of actinomycin D. RNAs were extracted and analysed by Northern blotting using full length 32P-labelled probes to the CAT coding sequence and to the S14 coding sequence as indicated. Lane numbers refer to h after addition of actinomycin D. (b) To verify whether the CAT activity reflected only the level of the different hybrid mRNAs or a more efficient recruitment and translation, the distribution of various hybrid CAT mRNAs among polyribosomal subfractions was estimated. HeLa cells were transfected with either pSV2CAT, pSV22-1 or pSV22-6 and CAT mRNAs were extracted after sucrose gradient fractionation of the ribosomes. The corresponding CAT cDNAs were then quantified by competitive PCR. The results shown in Fig. 5 do not show significant differences in the relative distribution of CAT mRNA whether or not they contain the 5'UTR of ICP22. Deletions in the 5'UTR of ICP22 were without effect on the distribution of CAT mRNA among the polyribosomal subfractions. Regardless of the plasmid transfected, approximately 50% of the CAT mRNAs were detected in the fraction containing the 60S subunits were probably present because of contamination with 80S ribosomes after sucrose gradient fractionation. There was no significant difference between the polyribosomal

No preferential recruitment of hybrid CAT mRNA by the translational apparatus

HeLa cells were transfected with either pSV2CAT, pSV22-1 or pSV22-6 and CAT mRNAs were extracted after sucrose gradient fractionation of the ribosomes. The corresponding CAT cDNAs were then quantified by competitive PCR. The results shown in Fig. 5 do not show significant differences in the relative distribution of CAT mRNA whether or not they contain the 5'UTR of ICP22. Deletions in the 5'UTR of ICP22 were without effect on the distribution of CAT mRNA among the polyribosomal subfractions. Regardless of the plasmid transfected, approximately 50% of the CAT mRNAs were found in fractions below a coefficient of sedimentation of 60S and 28 to 32% were associated with the ribosomes and polyribosomes. CAT mRNAs detected in the fraction containing the 60S subunits were probably present because of contamination with 80S ribosomes after sucrose gradient fractionation. There was no significant difference between the polyribosomal
distribution of the various CAT mRNAs tested. This indicates that the 5'UTR of ICP22 mRNA does not significantly influence the recruitment of CAT mRNA by the translational apparatus.

Discussion

We examined the involvement of the sequence coding for the 5'UTR of ICP22 mRNA as well as the role of the 5'UTR itself in expression of a CAT reporter gene under the control of the SV40 early promoter-enhancer. Our results show that replacement of the sequence coding for the 5'UTR of the CAT mRNA by the 513 bp sequence coding for the full-length 5'UTR and the first 16 bp of the translated region of ICP22 mRNA induces a 13-fold stimulation of CAT expression. Use of deletion mutants in this sequence shows the existence of an internal sequence which is important for expression of the hybrid CAT reporter gene. The stimulation appears to be due to specific elements located between NruI and EcoNI restriction sites within the sequence coding for the 5'UTR of ICP22 mRNA. Precise quantification of CAT mRNA revealed that the sequence coding for the 5'UTR induces an increase in the amount of the transcript. However, deletion of the sequence located between the NruI and EcoNI restriction sites reduces the amount of the transcript. Moreover, elimination of the sequence coding for the intron had no significant effect on the CAT activities or on the amount of the corresponding CAT mRNA. Therefore, an internal sequence of 163 bp appears responsible for the observed stimulatory effect. At the most the sequences required for stimulation can be mapped to the DNA between the NruI and FokI sites, not including the intron sequences. Results obtained after actinomycin D treatment show that the 5'UTR of ICP22 mRNA does not increase the amount of CAT mRNA by increasing the stability of the hybrid transcript. Indeed, CAT mRNA transcribed from plasmids with or without the sequence coding for the 5'UTR of ICP22 mRNA are degraded at the same rate, as revealed by their very similar half-lives. In addition, there is no significant difference in the relative nucleo-cytoplasmic distribution of the CAT transcripts whether or not they contain the 5'UTR of ICP22 mRNA. Therefore, an increase in the amount of CAT mRNA cannot be the result of an increase in the ICP22-CAT mRNA stability, or of preferential transportation of the transcripts containing the 5'UTR of ICP22 mRNA. In addition, the recruitment of the CAT mRNA by ribosomes is not influenced by the 5'UTR of ICP22 mRNA. Moreover, activity of the CAT enzyme appears proportional to the amount of the mRNA encoding it. Therefore, the increase in the amount of cytoplasmic CAT mRNA appears sufficient to fully account for the increase in the level of CAT expression. This suggests that the stimulation of CAT gene expression is mainly due to a transcriptional stimulation.

Some 5'UTR of β, γ1 and γ2 HSV mRNAs have been studied by others (Blair et al., 1987; Coen et al., 1986; Guzowski & Wagner, 1993; Huang et al., 1993; Pederson et al., 1992; Romanelli et al., 1992; Tedder et al., 1989; Weir & Narayan, 1990). Sequences coding for these 5'UTR efficiently stimulate gene expression in different ways. For example, the 5'UTR of the VP16 mRNA stabilizes the mRNA, whereas in the VP5 and UL38 mRNA the sequence regulates the amount of a reporter gene mRNA without affecting its stability (Guzowski & Wagner, 1993; Huang et al., 1993). These results suggest that different mechanisms are involved in the stimulation of gene expression by the different 5'UTR of HSV-1 mRNA or by the sequence encoding them. In the absence of viral infection the sequence encoding the 5'UTR of ICP22 mRNA increases the amount of the transcripts, thus enhancing gene expression under the control of a heterologous promoter. The characterization of the cellular factor(s) mediating this stimulation is of primary importance for understanding the early stage of the viral cycle. It is also possible that viral factor(s) modulate the effect of the 5'UTR of ICP22 mRNA, thus conferring additional advantages on gene expression subsequent to viral infection. Indeed, in ras-transformed cells or in neomycin-treated cells, immediate early gene expression is critical for productive infection and very sensitive to alterations of the mitogenic pathway (Garcin et al., 1990; Massé et al., 1990a).

For a few other viruses, the 5'UTR of viral mRNA has been implicated in the control of gene expression (Cullen, 1990; Degnin et al., 1993; Garfinkel & Katze, 1992; Geballe & Gray, 1992; Huang & Schneider, 1991; Meerovitch et al., 1991). Some of them play a role in the translational discrimination between viral and cellular mRNA. Post-transcriptional mechanisms allowing the differential expression of genes can involve a competition between mRNA for the translational apparatus. This implies that the viral mRNAs contain specific sequences that specifically increase their recruitment in the polyribosomal fraction. But the differential regulation of expression of endogenous and transduced genes during infection with a HSV-1 recombinant indicates, conversely, that preferential expression of HSV genes can be mediated through mechanisms that are independent of specific RNA sequences (Smibert & Smiley, 1990). In our conditions, no translational advantage was conferred to the chimeric RNA by the presence of the viral 5'UTR. However, this might not be the case in a competition assay, under conditions where translation is limiting (Lindquist & Petersen, 1990). In addition, regulation of gene expression at the transcriptional and/or post-
transcriptional levels occurs during HSV infection (Harris-Hamilton & Bachenheimer, 1985; Krikorian & Read, 1991; Orostar & Read, 1989; Roizman & Sears, 1993; Weinheimer & McKnight, 1987; Yager et al., 1990). Even if the HSV-1 α genes can be transcribed immediately after infection in absence of de novo protein synthesis, viral proteins are required for their maximal expression (Elshiekh et al., 1991). It has been previously shown that HSV-1 infection induces modifications of the translational apparatus (Garcin et al., 1990; Massé et al., 1990a, b). To verify whether viral factors could modulate the effect of the 5'UTR, in vivo and in vitro systems during infection or during cellular stress will be used.

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