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Characterization of the Products of the U_L43 Gene of Herpes Simplex Virus 1: Potential Implications for Regulation of Gene Expression by Antisense Transcription

KARA L. CARTER, PATRICIA L. WARD, AND BERNARD ROIZMAN*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

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The products, RNA or proteins, of the herpes simplex virus 1 open reading frame U_L43 have not been previously identified. The expression of an open reading frame antisense to U_L43, $U_L43.5$ (P. L. Ward, D. E. Barker, and B. Roizman, J. Virol. 70:2684–2690, 1996), has been reported. We report the existence of a transcript corresponding to the domain of the U_L43 open reading frame extending approximately 30 bp from the predicted TATA box to the predicted polyadenylation signal. The RNA of the U_L43 open reading frame accumulates to higher levels in the presence of phosphonoacetic acid, an inhibitor of viral DNA synthesis, than in its absence, whereas the $U_L43.5$ transcript accumulates in larger amounts in the absence of phosphonoacetic acid. The open reading frame tagged with a sequence encoding a 20-amino-acid epitope yielded a protein with an apparent M_r of 32,000, i.e., considerably lower than that predicted from the size of the open reading frame. The discovery of a pair of antisense genes expressed during productive infection raises the possibilities that additional antisense genes exist and that the antisense arrangement provides still another mechanism for regulation of gene expression.

McGeogh et al. identified 72 open reading frames (ORFs) of the herpes simplex virus 1 (HSV-1) genome on the basis of (i) prior mapping of viral transcripts and temperature-sensitive lesions and (ii) the presence of sequences which could potentially signify transcription initiation, transcription termination, and suitable codon usage within the ORF (17). Although in the subsequent 10 years many additional genes have been added to the list (1–4, 7, 14, 15, 27, 29), only one of the ORFs identified by McGeoch et al. (17) as a gene has remained an orphan, i.e., bereft of a known product. This ORF is U_L43.

Several reports are relevant to the studies of this ORF. The first, by Frink et al. (6), was a detailed analysis of the mRNA homologous to the *Hin*dIII L fragment of HSV-1 strain F [HSV-1 (F)]. These studies led to the detection of two transcripts which mapped to the strand antisense to U_L43, but neither of these transcripts corresponds to $U_L43.5$ and none were reported for U_L43. In the second study, MacLean et al. showed that the U_L43 ORF was dispensable for growth in cell culture, but attempts to demonstrate the presence of a gene product by its reactivity with polyclonal antibody to a peptide component of the proposed U_L43 protein were not successful (16). The third study, by Ward et al. (26), showed that an ORF antisense to the U_L43 gene is expressed. Lastly, the U_L43 ORF is conserved among the *Alphaherpesviridae* subfamily of herpesviruses. In one case, that of pseudorabies virus-infected cells, a transcript but not the protein product of the U_L43 homolog has been reported (22).

In this paper, we report that the U_L43 ORF is expressed. The results are of particular interest because U_L43 and $U_L43.5$ are both expressed, albeit at different times after infection. This is the first pair of antisense genes shown to be expressed during productive infection by wild-type HSV-1. Studies on the expression of these genes may yield insight to the process by which antisense genes are expressed.

* Corresponding author. Mailing address: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th St., Chicago, IL 60637. Phone: (312) 702-1898. Fax: (312) 702-1631.

MATERIALS AND METHODS

Cell lines and viruses. HSV-1(F) is the prototype virus used in this laboratory (5). The structures of the recombinant viruses R7119 and R7115 were reported elsewhere (26). R7119 lacks the U_L43 and $U_L43.5$ genes, whereas R7115 lacks the U_L43, $U_L43.5$, and U_L44 genes. Stocks of HSV-1(F) were made in HEp-2 cells; stocks of recombinant viruses were made and titrated in Vero cells. Vero and HEp-2 cell lines were from the American Type Culture Collection. The human 143 thymidine kinase-deficient ($143TK^-$) cells were obtained from Carlo Croce. Rabbit skin cells were originally obtained from J. McClaren. The cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with either 5% newborn calf serum (rabbit skin, Vero, or HEp-2 cells) or 5% fetal calf serum ($143TK^-$ cells).

Construction of plasmids. Restriction enzymes were obtained from New England Biolabs, Beverly, Mass. T4 ligase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The 6.6-kbp *Bam*HI I fragment of HSV-1(F) was cloned into the *Bam*HI sites of pGEM3Z and designated pRB172. A 2.3-kbp *Sph*I-*Nhe*I fragment containing the entire U_L43 ORF was cloned into the *Sph*I and *Xba*I sites of pGEM3ZF+. This plasmid, designated pRB4172, was digested with *Nae*I, and a 60-bp DNA oligomer encoding a cytomegalovirus (CMV) epitope (15) was cloned in frame with the U_L43 gene. This DNA oligomer contained a restriction site for *Kpn*I near the 3′ end to facilitate identification of clones containing the insert. The resultant plasmid, pRB4173, was sequenced to verify that the tag was in frame and intact. pRB4173 was then digested with *Pst*I and *Bam*HI (in the polylinker), and this 500-bp fragment was replaced with a 1.8-kbp *Pst*I-*Bam*HI fragment of HSV-1(F) *Bam*HI-I DNA. This plasmid, designated pRB4242, contains the entire U_L43 gene with the CMV epitope insertion and the entire U_L44 gene encoding the HSV-1 glycoprotein C (gC). pRB4530 was generated by collapsing a 5,629-bp fragment from pRB172, using the restriction endonucleases *Not*I and *Kpn*I. This fragment was then treated with T4 DNA polymerase to obtain flush ends and religated. pRB4740 was generated by liberating the 1,420-bp fragment of pRB4530 by using restriction endonuclease *Eco*RI and ligating it into the *Eco*RI site in the polylinker of pGEM3ZF+. pRB4443 contains the *Bam*HI-to-*Sac*I fragment of the α27 gene in pGEM3ZF+.

Preparation of RNA. 143TK cells were exposed to 10 PFU of the indicated virus per cell in the presence or absence of 300 μg of phosphonoacetate (PAA; Sigma) per ml and harvested at 12 h after infection. Cytoplasmic RNA was extracted as previously described (25) and digested with amplification-grade DNase (Gibco-BRL) according to the manufacturer's instructions. For purification of $poly(A)^+$ RNA, 143TK cells were grown in roller bottles for non-PAA-treated RNA or in three 150-cm^2 dishes for PAA-treated RNA. Total cellular RNA was extracted as previously described (23). One roller bottle or three 150-cm^2 dishes worth of RNA was applied to a single Magnabead $poly(A)^+$ selection column (Promega), and $poly(A)^+$ RNA was selected as instructed by the manufacturer.

RT-PCR. Twenty micrograms of cytoplasmic RNA was treated with DNase I as instructed by the manufacture (Gibco-BRL). Following DNase I treatment,

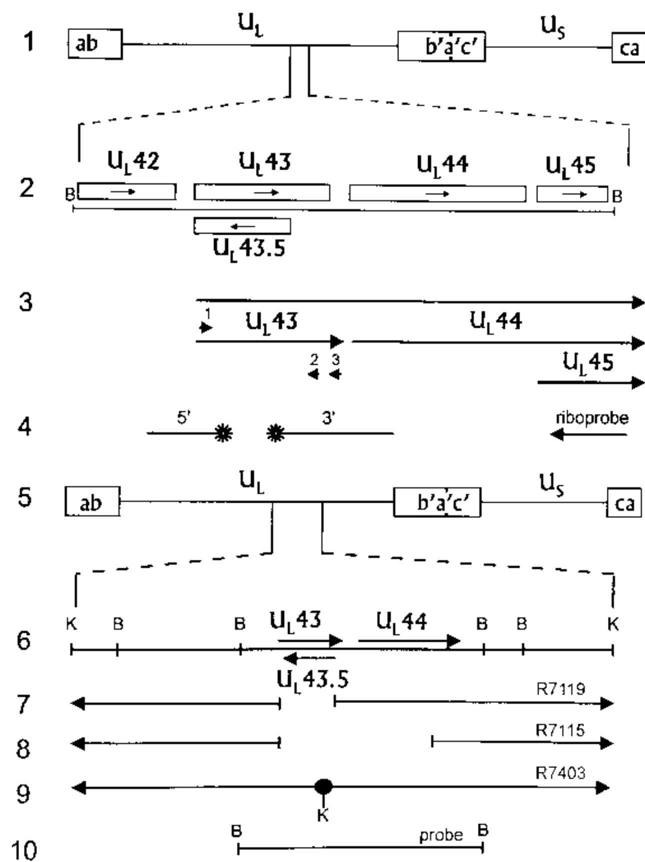

FIG. 1. Schematic diagram showing the sequence arrangement of wild-type and recombinant viruses, the domain of the HSV-1 genome containing the U_L43 ORF, and locations of the probes used in these studies. Line 1 represent the linear HSV-1(F) genome. Line 2 is an expansion of the region of the genome containing the U_L43 ORF. The U_L43 ORF is flanked by and has the same polarity as the U_L42 and U_L44 ORFs. $U_L43.5$ ORF is almost entirely antisense to the U_L43 ORF. Line 3 shows the locations of the transcripts in the domain shown in line 2. The small arrowheads designated 1 to 3 represent the map locations of oligonucleotides used in the RT-PCR and for hybridization to electrophoretically separated mRNA described in the text. Line 4 shows the map locations of the larger probes used in the RNA analyses. The lines ending with starbursts represent 5′ and 3′ probes labeled at the marked terminus. The riboprobe represents a strand-specific probe for detection of electrophoretically separated, denatured mRNA. Line 5 is again a linear representation of the HSV-1(F) genome. Line 6 is an expansion of the *Kpn*I fragment containing the U_L43 gene. The locations of the U_L43, U_L44, and $U_L43.5$ ORFs are indicated. Line 7 is a representation of the recombinant virus R7119 from which U_L43 and of $U_L43.5$ genes had been deleted. Line 8 is a schematic representation of the recombinant virus R7115 from which U_L43, $U_L43.5$, and U_L44 had been deleted. Line 9 is a schematic representation of the recombinant virus R7403 that contains an in-frame CMV epitope inserted in the U_L43 gene at a site represented by the closed oval. The CMV epitope insertion contains a *Kpn*I restriction endonuclease cleavage site. Line 10 shows the *Bam*HI fragment used as a probe in the Southern analyses. Abbreviations: K, *Knp*I; B, *Bam*HI.

100 μg of reverse primer 5′ATCGCCCGACCGCCCGCCCGTTGACCCCGG3′ (oligonucleotide 3 [Fig. 1, line 3]) was added; the mixture was incubated at 95°C for 5 min and then quickly chilled on ice. A solution of 1 mM deoxynucleoside triphosphates (dNTPs; Pharmacia), 40 U of RNasin (Promega), reverse transcription-PCR (RT-PCR) buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$), and 200 U of mouse mammary tumor virus reverse transcriptase (Gibco-BRL) was added, and the reaction mixture was incubated at 42°C for 1 h. Control reactions were treated with RNase A prior to the addition of the dNTPs, RNasin, buffer, and reverse transcriptase. To amplify the cDNA product, 100 μg of forward primer 5′GTTCTGTGCAGTCTCGGGGAGCCCGGCACG3′ (oligonucleotide 1 [Fig. 1, line 3]), 10% dimethyl sulfoxide, and 5 U of Amplitaq thermostable polymerase (Perkin-Elmer) were mixed with the products of the reverse transcriptase reaction. PCR was carried out for 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The PCR products were treated with RNase A prior to separation on a 1% agarose gel.

Analyses of denatured electrophoretically separated RNA. Poly(A)$^+$-selected RNA from one roller bottle or three 150-cm^2 dishes was loaded per lane and separated in a 1.5% agarose gel containing 6% formaldehyde. The RNA was then transferred to a Zeta-probe membrane (Bio-Rad) in 10× SSC (1.5 M NaCl plus 0.15 M trisodium citrate). The blots were air dried and baked at 80°C in a desiccating oven. Oligonucleotide probe 2, with the sequence 5′AGAGGCCCCGAAGCGTCGCCGCCAACCGGG3′, was radiolabeled at its 5′ end by polynucleotide kinase and [γ-^{32}P]ATP. The oligonucleotide was synthesized on an Applied Biosystems model 308B DNA synthesizer. All hybridization was done at 65°C overnight in hybridization solution (1 mM EDTA, 0.5 M NaH_2PO_4 [pH 7.2], 7% sodium dodecyl sulfate). The blot was stripped as instructed by the manufacturer (Bio-Rad). Plasmids pRB3910 encoding U_S11 (24) and pRB4443 encoding α27 were nick translated with [α-^{32}P]dCTP as instructed by the manufacturer (Promega).

S1 analysis. Twenty micrograms of cytoplasmic RNA was hybridized with either the 5′- or the 3′-specific probe in hybridization solution [80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM disodium piperazine-*N*,*N*′-bis(2-ethane sulfonic acid (PIPES; pH 6.4)]. The 5′ probe was generated by gel purification of the *Sal*I endonuclease fragment from pRB172. The purified fragment was then treated with calf intestinal phosphatase (United States Biochemical) to remove the 5′ phosphate. The dephosphorylated fragment was 5′ end labeled with [γ-^{32}P]ATP (Amersham) and polynucleotide kinase. The 3′ probe was generated by gel purification of the *Eco*RI endonuclease fragment from pRB4740. The cleavage left a 3′ recessed end whose next templated nucleotide would be a cytosine. The fragment was 3′ end labeled with [α-^{32}P]dCTP (Amersham) and the Klenow fragment (Boehringer Mannheim). The hybridization was carried out overnight at 58°C. To the reaction were added a 10-fold volume of ice-cold digestion buffer (250 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, 30 mM sodium acetate [pH 4.6]) and 200 U of S1 nuclease (Boehringer Mannheim). The digestion was carried out at 45°C for 30 min. The reaction mixture was then extracted with a mixture of phenol and chloroform and precipitated in 95% ethanol. The resuspended products were separated on a 6% polyacrylamide–7 M urea gel. Control reaction mixtures contained no input RNA.

Construction of R7403. Rabbit skin cells were cotransfected with viral DNA made from R7115-infected cells and plasmid pRB4242. Progeny virus was serially diluted and plated onto Vero cell monolayers for analysis of surface gC expression by black plaque assay (see below). DNA of purified recombinant viruses was analyzed by Southern blotting for the presence of both the CMV epitope tag and an intact *Bam*HI I fragment.

Black plaque assay. The procedures for the black plaque assay were essentially as previously described (12). Briefly, Vero cell monolayer cultures were exposed to serially diluted mixtures of the transfection mixture. The cells were kept at 37°C for 48 h and then incubated in blocking medium consisting of medium 199 supplemented with 1% heat-inactivated horse serum (199V) for 1 h at room temperature. The cultures were then exposed to monoclonal antibody HC1, specific for the viral gC (20), diluted 1:500 in 199V for 2 h at room temperature. Following three washes with 199V, cultures were exposed to a 1:500 dilution of biotinylated anti-mouse immunoglobulin G for 1 h at room temperature. The cultures were washed again three times and reacted with avidin bound to biotin-conjugated horseradish peroxidase (Vector Laboratories), and the color reaction was developed by using 1-chloro-4-naphthol substrate as previously described (12).

Purification and analysis of viral DNA. Viral DNA intended for transfections was prepared from potassium acetate gradients as previously described (9). Otherwise, viral DNA was purified by phenol-chloroform extraction of cytoplasmic extracts of infected Vero cells.

Polyacrylamide gel electrophoresis and immunoblotting. Infected-cell lysates were electrophoretically separated in denaturing 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, electrically transferred to a nitrocellulose sheet, soaked in a solution of 5% milk (Carnation) in phosphate-buffered saline (PBS), reacted with a 1:500 dilution of CMV antibody for 2 h at room temperature, and rinsed three times in PBS containing 1% Tween 20. The blots were reacted with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (1:3,000 dilution) for 1 h at room temperature and rinsed three times, and the reaction was developed by using reagents in an enhanced chemiluminescence kit purchased from Amersham Life Sciences.

RESULTS

The U_L43 ORF is transcribed. The DNA sequences surrounding the U_L43 ORF contain a TATA box and a polyadenylation signal (17). However, previous studies of this region of HSV-1 DNA failed to detect a transcript corresponding to the U_L43 ORF (6). We reasoned that a U_L43 transcript may be difficult to detect in the infected cell because it is either transcribed at a low level or has a high turnover rate or because of the existence of another gene, $U_L43.5$ (26), which lies com-

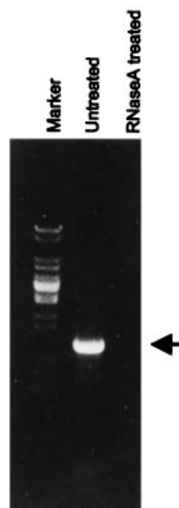

FIG. 2. Photograph of the DNA bands produced by RT-PCR designed to detect the presence of the U_L43 transcript. Cytoplasmic RNA extracted 12 h after infection of 143TK$^-$ cells with HSV-1(F) was reacted with a reverse primer (oligonucleotide 3) and extended with mouse mammary tumor virus reverse transcriptase for 1 h at 42°C in the presence of dNTPs. A forward primer (oligonucleotide 1) and a thermostable DNA polymerase were then added, and the reaction was cycled 40 times. The amplified products were separated on a 1% agarose gel. Lanes: Marker, DNA digested with restriction endonuclease *Bst*EII; Untreated, RT-PCR of cytoplasmic RNA; RNase A treated, RT-PCR of cytoplasmic RNA that had been treated with RNase A prior to the reverse transcription reaction.

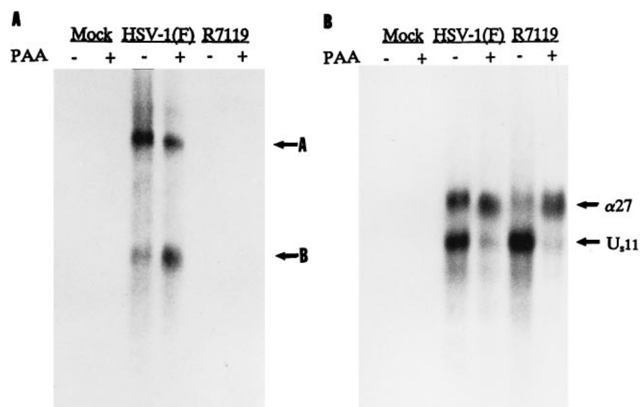

FIG. 3. Autoradiographic image of labeled probes hybridized to electrophoretically separated poly(A)$^+$-selected total RNA from cells harvested at 12 h after infection with the indicated viruses. (A) Accumulation of the U_L43 (band B) and readthrough (band A) transcripts. Poly(A)$^+$-selected RNA from one roller bottle of 143TK$^-$ cells was loaded in each lane. Lanes: Mock, RNA extracted from mock-infected cells; HSV-1(F), RNA extracted from HSV-1(F)-infected cells; R7119, RNA extracted from the U_L43 deletion virus R7119. PAA was present (+) or absent (−) in the medium during infection. (B) The same blot as in panel A, stripped and reprobed with α27 and U_S11-specific DNA probes.

pletely antisense to U_L43. Expression of the $U_L43.5$ gene could lead to the presence of double-stranded RNA which may be more subject to degradation by cellular enzymes (19) or hinder the detection of the U_L43 transcript by conventional mapping techniques that require hybridization. Therefore, we used RT-PCR to amplify specifically products arising by transcription of the U_L43 gene. Twenty micrograms of DNase I-treated cytoplasmic RNA extracted from HSV-1(F)-infected cells was reacted with reverse transcriptase and DNA primer 3, designed to anneal to sequences at the 3′ end of the putative U_L43 transcript (Fig. 1, line 3). Amplification of this cDNA by using primer 1 (Fig. 1, line 3), which has sequence identity with the 5′ end of the sense strand of the U_L43 ORF, resulted in the production of a 900-bp fragment (Fig. 2). This product was not due to the presence of contaminating DNA because digestion of the RNA with RNase A prior to reverse transcription precluded reverse transcription and amplification of the product (Fig. 2). We conclude that the U_L43 gene is transcribed during lytic infection.

To determine the approximate size of the U_L43 transcript, we analyzed RNA electrophoretically separated in denaturing agarose gels under conditions which increased the sensitivity of the assays. First, poly(A)$^+$ RNA extracted from an entire roller bottle of cells (2×10^8 to 4×10^8) was loaded per lane of an agarose gel. Additionally, in the expectation that the antisense transcript of the $U_L43.5$ gene interferes with the accumulation of the U_L43 mRNA, poly(A)$^+$ RNA was also extracted from three 150-cm^2 flask cultures infected and maintained in the presence of PAA in order to reduce the presence of the transcript of $U_L43.5$, a γ_2 gene (26). The electrophoretically separated RNAs were hybridized with radiolabeled oligonucleotide probe 2, specific for the 3′ end of U_L43 (Fig. 1, line 3). The U_L43 transcript was determined to be approximately 1.3 kb (Fig. 3A, arrow B) relative to the migration of rRNA (data not shown) and was not detected in RNA extracts of cells infected with the mutant virus R7119 (Fig. 3A), which lacks both the U_L43 and $U_L43.5$ genes (Fig. 1, line 7). The U_L43 transcript accumulated to higher levels in cells infected and maintained in the presence of PAA (Fig. 3A). This finding suggests that either U_L43 is regulated as a β gene (8) or expression of the $U_L43.5$ gene, which is blocked by PAA, inhibits the accumulation of the U_L43 transcript. To ensure that there were equal amounts of RNA present in all of the infected-cell RNA lanes, the blot in Fig. 3A was stripped and reprobed with nick-translated DNA fragments specific for the α27 and U_S11 transcripts. The α27 transcript was present in equal amounts in HSV-1(F) PAA+ and PAA− lanes and in slightly lower levels in PAA− than on PAA+ R7119 lanes (Fig. 3B). The effects of PAA on late gene expression may be inferred from the decreased levels of the U_S11 transcript in the PAA+ lanes of both HSV-1(F) and R7119 RNA extracts (Fig. 3B).

The oligonucleotide probe which hybridized to the U_L43 transcript also hybridized to a 4-kb transcript (Fig. 3A) which is absent from extracts of cells infected with R7119 (Fig. 3). A transcript of identical migration was similarly observed with a probe hybridizing with the 3′ end of the U_L44 transcript (data not shown). These two results suggest that this transcript begins at the U_L43 promoter, reads through the U_L43 polyadenylation signal, and terminates at the U_L44 polyadenylation signal.

Mapping of the 5′ and 3′ ends of the U_L43 transcript. An S1 nuclease protection assay was used to map the precise 5′ and 3′ termini of the U_L43 transcript. To map the 5′ end of the U_L43 transcript, the *Sal*I endonuclease fragment was cleaved from pRB172 and gel purified. The 5′ end of the fragment was dephosphorylated and labeled with [γ-^{32}P]ATP to generate the 5′ S1 probe (Fig. 1, line 4). This probe was then hybridized in solution with 20 μg of cytoplasmic RNA extracted from 143TK$^-$ cells infected with HSV-1(F) and maintained in the absence or presence of PAA for 12 h. After digestion with S1 nuclease, the products were separated on a 6% polyacrylamide–7 M urea gel. Protected products migrating with the 90-bp marker were seen in RNA extracts of cells infected in the presence of PAA (Fig. 4A). These protected species were not present in reactions with RNA extracted from non-PAA-

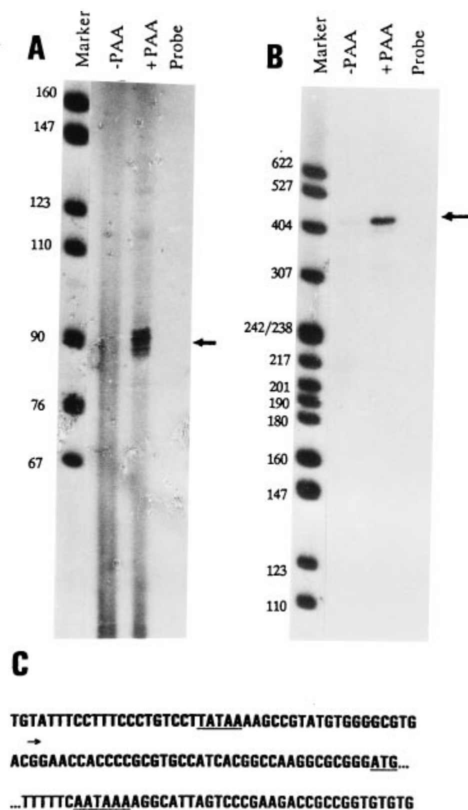

FIG. 4. Autoradiographic images of the probes and of the products of S1 analyses of the 3′ and 5′ ends of the U_L43 transcript. Twenty micrograms of cytoplasmic RNA extracted 12 h after infection with HSV-1(F) was used per reaction. Lanes: Marker, pBR322 DNA digested with restriction endonuclease *Msp*I and 3′ end labeled with [α-^{32}P]dCTP by using the Klenow fragment; −PAA, S1 digest of cytoplasmic RNA from cells infected in the absence of PAA; +PAA, S1 digest of cytoplasmic RNA from cells infected in the presence of PAA; Probe, S1 digest of the probe alone with no input RNA. (A) Results of analyses of the 5′ end of the U_L43 transcript; (B) results of analyses of the 3′ end of the U_L43 transcript; (C) nucleotide sequences encompassing the mapped initiation and termination sites of transcription. The starting nucleotide corresponds to 94714 and the ending nucleotide corresponds to 96099 of the prototypic HSV-1 genome. The proposed TATA box, translation initiation codon, and polyadenylation signal are underlined. The proposed start site of transcription is indicated by an arrow. Sizes in panels A and B are indicated in nucleotides.

treated cells or in reaction mixtures to which no RNA was added (Fig. 4A). These results placed the initiation site of the U_L43 transcript approximately 30 bases downstream from a consensus TATA-box element and upstream of the start site of the U_L43 ORF proposed by McGeogh et al. (17).

To map the 3′ end of the U_L43 transcript, the *Eco*RI endonuclease fragment was cleaved from pRB4740, gel purified, and labeled at the 3′ end with [α-^{32}P]dCTP (Fig. 1, line 4). The probe was hybridized in solution with 20 μg of cytoplasmic RNA from 143TK$^-$ cells infected with HSV-1(F) and maintained in the absence or presence of PAA for 12 h. After digestion with S1 nuclease, the products were separated on a 6% polyacrylamide–7 M urea gel. Figure 4B shows a single protected species migrating slightly more slowly than the 404-bp marker in RNA extracts of both untreated and PAA-treated cells, although the abundance was significantly increased in the presence of PAA. A reaction performed with the probe but no RNA did not produce the protected species (Fig. 4B). The size of the protected species suggests that the U_L43 transcript terminates at the polyadenylation site proposed by McGeogh et al. (17).

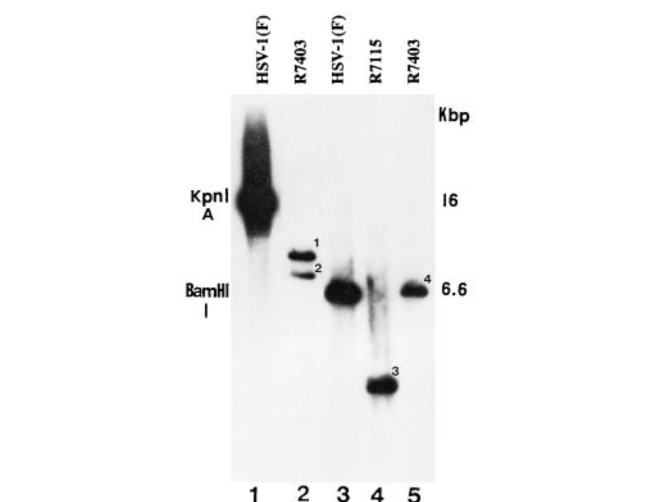

FIG. 5. Autoradiographic images of the electrophoretically separated restriction digests of the DNAs of HSV-1(F), R7403 (containing the CMV epitope tag in the U_L43 gene), and the deletion mutant virus, R7115. HSV-1(F) and R7403 DNAs were digested with *Kpn*I (lanes 1 and 2); HSV-1(F), R7403, and R7115 DNAs were digested with *Bam*HI (lanes 3 to 5). Digested DNAs were separated on a 1.0% agarose gel, transferred to a Zeta-probe membrane, and probed with ^{32}P-labeled *Bam*HI I fragment of HSV-1(F). Bands 1 and 2 represent the 9- and 7-kbp fragments generated by cleavage of the *Kpn*I site contained within the CMV epitope tag DNA sequence. Bands 3 and 4 represent the 3.9-bp fragment generated by deletion of 2.7 kbp from the *Bam*HI I fragment in R7115, which results in deletion of the U_L43, $U_L43.5$ and U_L44 genes, and the repair of the *Bam*HI I fragment in R7403, respectively. The HSV-1(F) 16-kbp *Kpn*I A and 6.6-kbp *Bam*HI I fragments are indicated.

Identification of U_L43 protein. Numerous attempts to generate polyclonal rabbit antisera to the native U_L43 protein have not been successful. We therefore constructed recombinant virus R7403, in which a CMV epitope tag was inserted into the U_L43 gene in order to identify the protein product. Rabbit skin cells were cotransfected with plasmid pRB4242 and intact, R7115 recombinant virus DNA. R7115 lacks both the U_L43 ORF and that of U_L44, which encodes gC. Progeny viruses were plated on Vero cell monolayer cultures, and plaques were analyzed for surface expression of gC by the black plaque assay. Recombinant viruses in which gC was detected were analyzed for the presence of the CMV epitope by hybridization to electrophoretically separated DNA digests. Viral DNAs made from HSV-1(F)- and R7403-infected cells were digested with *Kpn*I, electrophoretically separated on a 1.0% agarose gel, and transferred to nitrocellulose. The blot was probed with nick-translated HSV-1(F) *Bam*HI I fragment (Fig. 1, line 10). The probe hybridized to the approximately 16-kbp *Kpn*I A fragment of HSV-1(F) DNA (Fig. 5, lane 1). The CMV tag in R7403 contains a *Kpn*I restriction site resulting in the cleavage of *Kpn*I A into bands 1 and 2 (Fig. 5, lane 2). Repair of the deletion in R7115 DNA was verified in R7403 by the presence of a 6.6-kbp HSV-1(F) *Bam*HI I fragment (Fig. 5, lanes 3 to 5).

To assay for expression of the CMV-tagged U_L43 protein, 143TK$^-$ cells were mock infected or infected with HSV-1(F) or R7403. The lysates of cells harvested 20 h after infection or mock infection were electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with the CMV antibody. As shown in Fig. 6, a protein reacting with the CMV antibody was present in lysates of R7403-infected cells but not in lysates of mock- or HSV-1(F)-infected cells. The CMV-tagged protein migrates with an apparent M_r of 32,000. We estimate that the presence of the CMV epitope

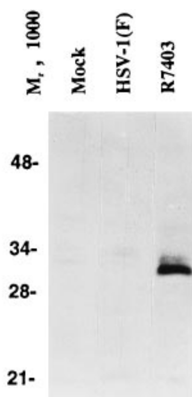

FIG. 6. Photograph of an immunoblot of electrophoretically separated lysates of infected 143TK⁻ cells probed with the monoclonal antibody against the CMV epitope tag inserted into the U_L43 gene in R7403.

increases the apparent M_r by 2,000, and therefore the apparent M_r of U_L43 protein deduced from its electrophoretic mobility is 30,000. This estimate is approximately 10,000 to 15,000 smaller than would be predicted from the size of the U_L43 ORF.

DISCUSSION

In this report, we show that (i) the U_L43 gene is transcribed, (ii) the transcript of U_L43 maps to the initiation and termination sites proposed by McGeogh et al. (17), (iii) the U_L43 transcript was more readily detectable in RNA extracts from cells infected and maintained in the presence of PAA, and (iv) the U_L43 ORF generated a protein product with an apparent M_r of 30,000. The studies described in this report present three keys issues.

(i) Earlier studies have shown that late in infection, approximately 50% of the genome is represented in abundant symmetric transcripts and that the double-stranded RNA prepared from such transcripts has the melting temperature predicted of bona fide double-stranded RNA with the G+C content of HSV-1 DNA (10, 13). These observations could be accounted for in part by run-on transcripts resulting from improper termination. In recent years, several lines of evidence suggest that in fact symmetric transcription is not merely an accident. Specifically several reports indicate that the usage of polyadenylation signals is regulated (18). In addition, several HSV-1 genome domains are symmetrically transcribed in a regulated, reproducible fashion. Thus, the 8.3-kb latency-associated transcript is antisense to both $\alpha 0$ and $\gamma_1 34.5$ genes, and the abundant 2.0- and 1.5-kb latency associated transcripts which accumulate in nuclei of latently infected cells are antisense to the $\alpha 0$ gene (4, 21). More significantly, ORF P is almost completely antisense to the $\gamma_1 34.5$ gene (14). ORF P is repressed by ICP4, and in the course of productive infection with wild-type virus, ORF P and $\gamma_1 34.5$ would not be expected to be expressed simultaneously in the same cells (29). The possibility that transcription of ORF P is incompatible with simultaneous transcription of the $\gamma_1 34.5$ gene emerged from studies showing that transcription of the $\gamma_1 34.5$ gene was grossly reduced in cells infected with a mutant in which the ORF P gene was derepressed by mutagenesis of the ICP4 binding site (29). The question therefore arises as to whether there are additional gene pairs antisense to each other and how the antisense arrangement contributes to the mechanisms for regulation of gene expression.

(ii) In this and in an earlier report from this laboratory (26), we showed that both U_L43 and $U_L43.5$ genes are expressed. In wild-type virus-infected cells, accumulation of the $U_L43.5$ protein was reduced in the presence of PAA, whereas the amount of accumulated U_L43 RNA increased under the same conditions. Operationally, U_L43 is therefore defined as a β gene whereas $U_L43.5$ is defined as a γ gene. As a general principle, increased accumulations of β-gene expression in the presence of specific inhibitors of DNA synthesis could be inferred to be due to the absence of DNA templates which enable transcription of γ genes or to the absence of γ gene products which shut off β gene expression. Nevertheless, it is noteworthy that cells infected in the absence of PAA with the recombinant virus R7403 accumulate markedly higher amounts of the U_L43 transcript than in PAA-treated cells (data not shown). The recombinant R7403 carries a CMV epitope in frame with U_L43 ORF but has a stop codon inserted into the $U_L43.5$ ORF. One hypothesis that could explain the discrepancy between the results obtained with the wild-type parent and this recombinant is that insertion of the stop codon rendered the $U_L43.5$ mRNA less stable, thus enabling the accumulation of the U_L43 mRNA. However, this hypothesis cannot be confirmed because the $U_L43.5$ transcript has not been detectable by hybridization. The U_L43 transcript was also much more abundant in extracts from cells infected with the R7403 recombinant than in those of cells infected with HSV-1(F), suggesting that the presence of the $U_L43.5$ transcript either masks the detection of the U_L43 transcript or causes a reduction in its expression. An additional argument, that the U_L43 transcript is either less readily detectable or less abundant as a result of the presence of the $U_L43.5$ transcript, comes from the transcriptional analysis of U_L43 in cells infected with pseudorabies virus (22). The U_L43 transcript in such cells was relatively abundant, and there appears to be no $U_L43.5$ gene present in this virus.

The available data are not sufficient to discriminate between a general mechanism which shifts expression from β to γ genes and the specific instance in which the transcription of one ORF (e.g., $U_L43.5$) may interfere with the transcription of the antisense ORF (U_L43). The central question, whether viral gene expression is or can be regulated by antisense transcription, remains to be resolved.

(iii) The predicted amino acid sequence of the U_L43 protein suggests that it may contain seven transmembrane domains and that it is composed almost entirely of hydrophobic alpha helices. The extremely hydrophobic nature of this protein may explain why multiple attempts by this laboratory and others to generate antibody to the native U_L43 protein have failed (16). By tagging the gene, we have identified a protein product with an apparent M_r of 30,000. The M_r of this protein is approximately 10,000 to 15,000 smaller than the M_r estimated for the U_L43 ORF. The sequence predicts six methionines in frame with the U_L43 ORF, and the results of our RNA mapping studies suggest that the second methionine (beginning at nucleotide 94,799) is the initiation codon of the U_L43 gene. Furthermore, mutagenesis of this codon in the R7403 virus resulted in disappearance of the CMV-tagged protein by immunofluorescence (data not shown). We have sequenced most of the U_L43 ORF and found no stop codons that would account for this smaller size, nor have we been able to detect any posttranslational modification of the U_L43 protein. Immunofluorescence studies using R7403 and a mouse monoclonal antibody directed against the CMV epitope have yielded images suggesting that U_L43 protein localized in or at cell membranes (28).

The function of the U_L43 gene product remains to be elucidated. Deletion analysis of the U_L43 gene by this and other

laboratories failed to ascribe a specific function to the U_L43 gene (11, 16). The multiple hydrophobic stretches of the U_L43 protein are similar to those of some proteins that create channels in the plasma membrane. It is conceivable that U_L43 codes for a membrane channel protein whose function is necessary in some cells but not in those cell lines tested to date.

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