Characterization of the Human Cytomegalovirus *irs1* and *trs1* Genes: a Second Immediate-Early Transcription Unit within *irs1* Whose Product Antagonizes Transcriptional Activation

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We have characterized the *irs1* and *trs1* genes of human cytomegalovirus. The previously identified mRNAs as well as their corresponding protein products pIRS1 and pTRS1 could be detected during all phases of the viral replication cycle. The proteins were present in the nucleus and cytoplasm during the immediate-early and early phases of the viral growth cycle but were predominantly cytoplasmic late after infection. Although pIRS1 and pTRS1 exhibited little transcriptional activation potential on their own, both cooperated with the IE1 and IE2 proteins to enhance expression from a variety of viral promoters. We have also identified a previously undescribed immediate-early gene product encoded within the *irs1* gene that we have termed pIRS1[263]. This new protein is encoded within the 3′ end of the *irs1* gene and is in the same reading frame as the large pIRS1 protein. Expression of the *irs1[263]* gene is controlled by a promoter that resides within the *irs1* open reading frame in the unique short region of the viral genome. pIRS1[263] resides in the nucleus and antagonizes transcriptional activation by cytomegalovirus immediate-early proteins. We propose that pIRS1[263], whose promoter responds to immediate-early transcriptional activators, serves as part of a regulatory loop, antagonizing the function of the viral proteins that are responsible for its synthesis.

Human cytomegalovirus (HCMV) is a human pathogen with major medical consequences in immunodeficient and immunocompromised individuals (reviewed in references 1, 4, 20, and 28). Like other herpesviruses, HCMV expresses its genes in a temporally regulated manner. The first HCMV genes to be expressed in infected cells are the so-called immediate-early genes, the products of which are thought to be required for regulation of the early group of genes. Products of known early genes are necessary for viral DNA replication as well as for regulation of late gene expression. Proteins produced by the virus in the late phase of the replicative cycle include those required for virion assembly and egress from the cell.

The analysis of the HCMV sequence revealed the presence of two open reading frames that were contained partially within repeated sequences and partially in the unique sequences at either end of the short unique domain of the viral genome: *irs1* (also known as HQRF1) and *trs1* (also known as HHLF1) (27). *irs1* extends from an internal repeated sequence into the short unique domain, and *trs1* extends from a terminal repeated sequence into the short unique domain. As a result, synthesis of their mRNAs is controlled by identical repeated promoters and the two proteins share residues at their amino termini but differ in their carboxy-terminal sequences. It was shown that *trs1* mRNA is expressed in cycloheximide-treated cells, and therefore the gene was assigned to the immediate-early category of viral genes. Since *irs1* is regulated by the same promoter as *trs1*, it was expected that it too would be expressed as an immediate-early gene. Our studies have confirmed this prediction.

Studies of pIRS1 (846 amino acids [aa], 91 kDa) and pTRS1 (788 aa, 84 kDa) function have demonstrated that one of the two open reading frames is required for transient complementation of *ori*Lyt-dependent viral DNA synthesis (21, 22) and

that the *irs1* region is not essential for viral replication, as a deletion of this region did not appreciably change the pattern of viral growth in permissive cell lines (13). Transactivating properties of pIRS1 and pTRS1 were investigated in cotransfection experiments (11, 14, 26). Plasmids containing the *irs1* and *trs1* genes upregulated the activity of the late ICP36 promoter in the presence of the viral IE1 and IE2 proteins but did not transactivate it when transfected alone (26). It was also shown that pTRS1-expressing plasmids produced severalfold-higher levels of activity than pIRS1-expressing vectors did when the reporter gene was controlled by the UL54 promoter (14) and other early viral promoters regulating the expression of genes involved in viral replication (11).

In this paper, we present results of an analysis of the *irs1* and *trs1* gene products in infected cells and we show that pIRS1 and pTRS1 serve as coactivators with IE1 and IE2 proteins of promoters belonging to different temporally regulated classes of viral genes. We identify and characterize a new mRNA and its protein product, pIRS1[263], that is encoded by the *irs1* gene. Finally, we demonstrate that pIRS1[263], which is maximally expressed during the early phase of infection, can antagonize the transcriptional activating capability of pTRS1.

MATERIALS AND METHODS

Virus stock, cell lines, and viral infection. HCMV strain AD169 (24) was purchased from the American Type Culture Collection. Virus was propagated in human foreskin fibroblasts (HFFs) obtained from local hospitals. The cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Viral stocks were collected in medium from low-passage-number infected HFFs and frozen without further processing at −80°C. For immediate-early RNA, HFF cells were infected at a multiplicity of infection of 5 to 10 PFU/cell and incubated in the presence of 100 μg of cycloheximide per ml for 18 h. Early RNA was collected 24 h after infection of the HFF cells with HCMV in the presence of phosphonoacetic acid. For late RNA, infection was allowed to proceed for 84 to 96 h in the absence of any drug. Immediate-early proteins were harvested from cycloheximide-blocked HFF cells after a 2-h release from the drug during which cells were washed twice with phosphate-buffered saline (PBS) and once with prewarmed culture medium, incubated for 1 h at 37°C, and washed once again with a fresh medium.

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Cloning. The fragments encoding peptides specific to the C-terminal regions of pIRS1 [pET21d(+)-IRS1C; genomic nucleotide positions 191740 to 192302; aa 660 to 846] and pTRS1 [pET21d(+)-TRS1C; genomic nucleotide positions 226137 to 226691; aa 589 to 788] and the genomic region corresponding to the first 177 aa common to both pIRS1 and pTRS1 [pET21d(+)-T/IRS1; genomic positions 189764 to 190295 and 227948 to 228455] were amplified by means of *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, Calif.) directly from purified HCMV AD169 DNA. These fragments were cloned into pET21d(+) (Novagen, Madison, Wis.) for expression in *Escherichia coli*. Plasmids used for synthesis of single-stranded riboprobes were prepared by recloning the *Nhe*I-*Xho*I DNA fragments from pET21d(+) into the *Xba*I-*Xho*I sites of pSP72 (Promega Biotec, Madison, Wis.) and named pSP72-IRS1C, pSP72-TRS1C, and pSP72-T/IRS1. pSP72-*luc*, the plasmid used for the synthesis of antisense riboprobes specific for luciferase mRNA, was constructed by recloning the *Xho*I-*Xba*I fragment of pGL3-Basic into the *Xho*I and *Xba*I sites of pSP72. pSP72-*luc* was linearized with *Sph*I, and probes were synthesized by runoff transcription with the T7 RNA polymerase. Antisense riboprobes for detection of IE2 mRNA were synthesized from the pSP72-IE1ex3/4 vector, which was prepared by recloning a 936-bp *Sac*I-*Eco*RV fragment between genomic positions 172086 and 173021 from pCM1058 into the sites of pSP72. The plasmid was linearized with *Bst*1107I, and probes were produced by runoff transcription with the T7 RNA polymerase. pTRI-Human-Actin, used for the production of antisense riboprobes to human β-actin, was purchased from Ambion, Inc. (Austin, Tex.).

The *irs1*, *trs1*, and *irs1*[263] open reading frames were amplified with *Tth* polymerase (Clontech Laboratories, Palo Alto, Calif.) by using appropriate primers in accordance with the manufacturer's recommendations and cloned into the *Nhe*I-*Xho*I sites of pET21d(+) to produce pET21d(+)-IRS1, pET21d(+)-IRS1[263], and pET21d(+)-TRS1 for expression in BL21(DE3) cells (Novagen) and for in vitro transcription followed by translation in the reticulocyte system (Promega Biotec). The DNA fragments were then excised from these vectors with the *Xho*I and *Bgl*II restriction endonucleases and cloned into the *Xho*I and *Bam*HI sites of pCITE4b(+) (Novagen). This step added a six-His tag DNA sequence as well as a stop codon to the 3′ end of each open reading frame. The inserts were excised from the pCITE4b(+) vectors with *Nhe*I and *Bgl*II and cloned into the *Nhe*I and *Bam*HI sites of pCEP4 (Invitrogen, San Diego, Calif.) for expression in eukaryotic cells (pCEP4-IRS1-His, pCEP4-IRS1[263]-His, and pCEP4-TRS1-His).

The luciferase reporter plasmid, pGL3-HCMV-MIEP, containing a 880-bp DNA sequence of the HCMV major immediate-early promoter was constructed by recloning the *Sal*I-*Hin*dIII fragment of pCEP4 into the *Xho*I-*Hin*dIII sites of pGL3-Basic. The ICP36 promoter sequence was amplified with viral DNA between genomic positions 56540 and 57090 and cloned into the pGL3-Basic luciferase vector (Promega Biotec). The luciferase reporter, pGL3-IRS1[263], containing the putative *irs1*[263] promoter sequence and the *irs1*[263] Kozak consensus sequence (16, 17) replacing the Kozak consensus sequence of the luciferase gene was prepared by recloning the *Nhe*I-*Bsp*HI fragment from pET21d(+)-FL-IRS1 (~1.7 kb) into the *Nhe*I-*Nco*I sites of pGL3-Basic.

pCGN-IE1 and pCGN-IE2 have been described previously (29), and pHM142 was a gift from T. Stamminger (2).

For experiments in which the presence of introns within the *trs1* mRNA was investigated, two regions were amplified from cDNA: SL-5/3-1 (between positions 226117 and 226634) and SL-5/3-2 (between genomic positions 225948 and 226634). The amplifications were performed with *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.).

PCRs were typically performed as follows: denaturation at 94°C for 2 min (1 cycle); denaturation at 94°C for 1 min and annealing and extension at 68°C for 3 min (5 cycles); and denaturation at 94°C for 1 min, annealing at 58°C, and extension at 70°C (25 cycles), followed by 1 cycle at 70°C for 10 min. All products were synthesized on the 9600 GeneAmp PCR System (Perkin-Elmer Corp., Foster City, Calif.).

RNA analysis. Total-cell RNA was purified from HCMV-infected cells by means of the TRIzol reagent (Gibco/BRL, Gaithersburg, Md.) as recommended by the manufacturer. Total-cell RNA for reverse transcription was further digested for 30 min at 37°C with RNase-free DNase (Promega Biotec), extracted once with 1:1 phenol-chloroform and once with chloroform, and precipitated with 0.6 M sodium acetate and ethanol. Fractionation of RNA into cytoplasmic and nuclear fractions was performed as described previously (3, 9), but with the following modifications. Nonidet P-40 (0.1%) was used in the lysis buffer, cells were lysed for 5 min on ice and centrifuged for 2 min at full speed in a microcentrifuge at 4°C, and the nuclear fraction was treated rapidly with TRIzol and processed exactly as total-cell RNA. Polyadenylated RNAs were selected with poly(dT)-oligotex beads (Qiagen, Chatsworth, Calif.).

Riboprobes were prepared by runoff transcription as follows. For Northern blots, pSP72-IRS1C, -TRS1C, -TIRS1, and -IR151 were linearized with *Bam*HI. Antisense riboprobes of 557, 596, 531, and 310 nucleotides (nt), respectively, were transcribed from the SP6 promoter by the SP6 polymerase (Promega Biotec) and labeled to high specific activity with [α-^{32}P]UTP (Amersham, Arlington Heights, Ill.). Following the transcription reactions, antisense RNA was purified on QuickSpin G-50 Sephadex columns (Boehringer Mannheim).

Transfer of RNA onto nylon membranes (Schleicher & Schuell, Keene, N.H.) and subsequent blots were performed essentially as described previously (18). Samples contained 10 to 20 μg of total-cell RNA or 2 to 5 μg of poly(A)-selected RNA. One percent agarose gels were prepared in buffer A containing 20 mM morpholinopropanesulfonic acid (MOPS; pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. RNA samples were dissolved in buffer A containing 50% molecular-biology-grade formamide (Gibco/BRL), heated for 7 min at 60°C, and subjected to electrophoresis at 60 to 80 V for 3 to 4 h in buffer A. Following electrophoresis, the gel was stained with ethidium bromide to visualize the RNA markers and verify the integrity of the RNA. RNA was transferred for 12 to 16 h onto neutral Nytran nylon membranes in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) by means of a TurboBlotter (Schleicher & Schuell). The membranes were rinsed briefly in 2× SSC. RNA was cross-linked to the membranes with UV light by using a Stratalinker (Stratagene Cloning Systems). Membranes were prehybridized at 60 to 65°C for 4 to 6 h in a solution containing 50% formamide, 5× SSC, 1% sodium dodecyl sulfate, 5× Denhardt's solution, 50 mM sodium phosphate (pH 6.5), and 200 μg of *E. coli* tRNA per ml. Following prehybridization, an in vitro-transcribed antisense RNA probe was added directly to the solution (1.0×10^6 to 3.0×10^6 cpm/ml). The use of antisense RNA probes labeled to high specific activity necessitated high-stringency washes at 75 to 80°C in 0.1× SSC and 0.1% sodium dodecyl sulfate.

Production of monoclonal antibodies to pIRS1 and pTRS1. The fragments encoding peptides specific to the C-terminal regions of pIRS1 and pTRS1 were expressed from pET21d(+)-IRS1C and pET21d(+)-TRS1C in BL21 (DE3)pLysS cells (Novagen). For production of six-His-tagged fusion proteins, protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed by sonication 3 h postinduction, and fusion proteins were purified with nickel-nitrilotriacetic acid-agarose beads (Qiagen). Recombinant pIRS1C and pTRS1C proteins were diluted in PBS and injected at a concentration of 20 to 40 μg/0.1 ml in the RIBI adjuvant (1:1 volume of PBS-diluted recombinant viral polypeptide and adjuvant) into female BALB/c mice. Mice were bled after the third injection, and the titer of antibodies present was determined by enzyme-linked immunosorbent assay as indicated below. After the fourth booster injection, spleen cells of immunized mice were fused with SP2/0-Ag14 cells as described.

Enzyme-linked immunosorbent assays were used to detect pIRS1, pIRS1[263], and pTRS1-specific antibodies as described previously (6). Western blots with infected cell lysates and in vitro-translated proteins were performed as described previously (12).

Indirect immunofluorescence. At appropriate times after infection of HFF cultures on coverslips (29), the medium was removed and cells were rinsed twice with prewarmed PBS. Procedures that follow were performed at room temperature. Cells were fixed in freshly prepared 4% paraformaldehyde for 15 min, rinsed three times with PBST buffer (PBS containing 0.1% Triton X-100 and 0.05% Tween 20). Bovine serum albumin (1%) in PBST was used to block cells for 1 h. Monoclonal antibodies were used at a 1:10 dilution in PBST and incubated with cells for another hour. Cells were washed three times with PBST, incubated with fluorescein isothiocyanate-labeled secondary goat anti-(mouse immunoglobulin G-Fc) antibody (Sigma Chemical Co., St. Louis, Mo.) for 40 min, and then washed extensively with PBST. Cells were mounted in Aqua-Mount (Lerner Laboratories, Pittsburgh, Pa.) on glass slides and analyzed by confocal microscopy.

Cell transfections and luciferase assays. pGL3-ICP36, pGL3-IRS1[263], pGL3-HCMV-MIEP, and pHM142 were used as reporter plasmids. Either HeLa or HFF cells grown to ~60% confluency were transfected in triplicate by means of Lipofectamine as recommended by the manufacturer (Promega Biotec). Briefly, 2.5 μg of DNA was diluted in 100 μl of Opti-MEM I (Gibco/BRL) and mixed gently with 100 μl of Opti-MEM I containing 5 μl of Lipofectamine. DNA-Lipofectamine complexes were allowed to form for 30 min at room temperature. Eight hundred microliters of Opti-MEM I was then added to each tube. The contents of each tube were mixed gently and overlaid onto HeLa or HFF cells prerinsed with Opti-MEM I. After 11 h Opti-MEM I was removed and replaced with fresh Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. In all experiments the total amount of plasmid DNA was held constant by including vector plasmid carrying the HCMV major immediate-early promoter, but no insert, to balance the amount of effector plasmid in each reaction. Luciferase activity was analyzed 48 h posttransfection unless noted otherwise.

Transfected DNA and RNA analysis. HeLa cells were transfected as indicated above, and RNA as well as DNA was harvested 48 h posttransfection. DNA was separated on a 1% agarose gel. Southern blots were performed in the same manner as Northern blots except that sonicated herring sperm DNA was added to the hybridization buffer. Ten micrograms of transfected-cell DNA was used to analyze copy numbers of transfected pGL3-ICP36 and pCGN-IE2 plasmids with sense probes synthesized by the SP6 RNA polymerase from the pSP72-*luc* and pSP72-IE1ex3/4 vectors, respectively. Northern blots utilized 30 μg of total transfected-cell RNA, and reporter gene mRNA was detected with antisense riboprobes produced by the T7 RNA polymerase from the pSP72-*luc* vector.

RESULTS

A new mRNA species encoded within the *irs1* open reading frame. We began our studies of the *irs1* and *trs1* genes by analyzing the steady-state levels of their mRNAs. HCMV immediate-early RNA was isolated from infected HFF cultures
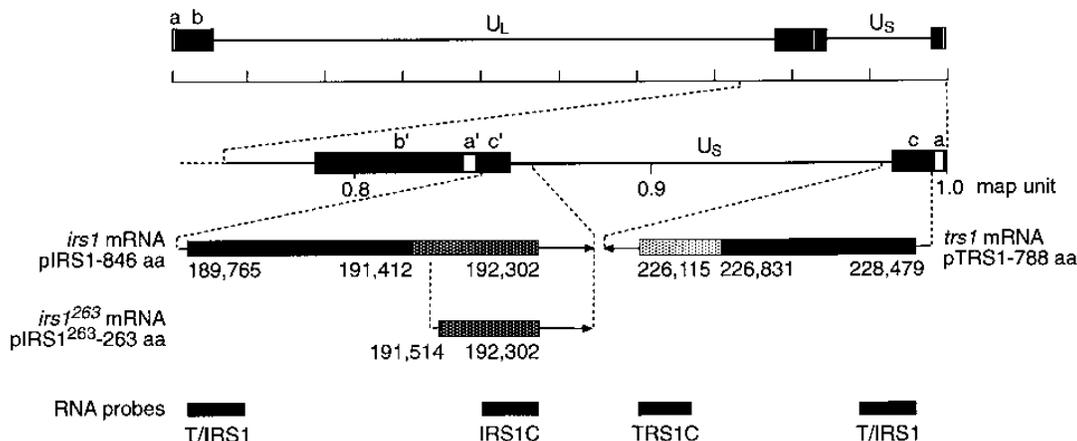
FIG. 1. Diagram of the HCMV genome showing the locations of the *irs1*, *irs1²⁶³*, and *trs1* open reading frames. The complete HCMV genome is represented at the top of the figure with repeated sequences (a, b, and c) and unique long (U_L) and unique short (U_S) domains indicated. pIRS1 and pTRS1 share 549 N-terminal aa. pIRS1²⁶³ comprises the 263 aa at the C terminus of pIRS1. Nucleotide sequence landmarks are identified according to the HCMV strain AD169 numbering of Chee et al. (7). RNA sequences used as probes for Northern blot analysis are identified at the bottom of the figure.

that were maintained in the presence of cycloheximide for 16 h, early RNA was isolated from infected cells maintained in the presence of phosphonoacetic acid for 24 h, and late RNA was prepared at 86 h after infection from cells that were not treated with drugs. Total, nuclear, and cytoplasmic RNAs were prepared from infected cells and analyzed by Northern blotting using a series of domain-specific probes (Fig. 1). *irs1* and *trs1* mRNAs were evident in the immediate-early RNA prepara-
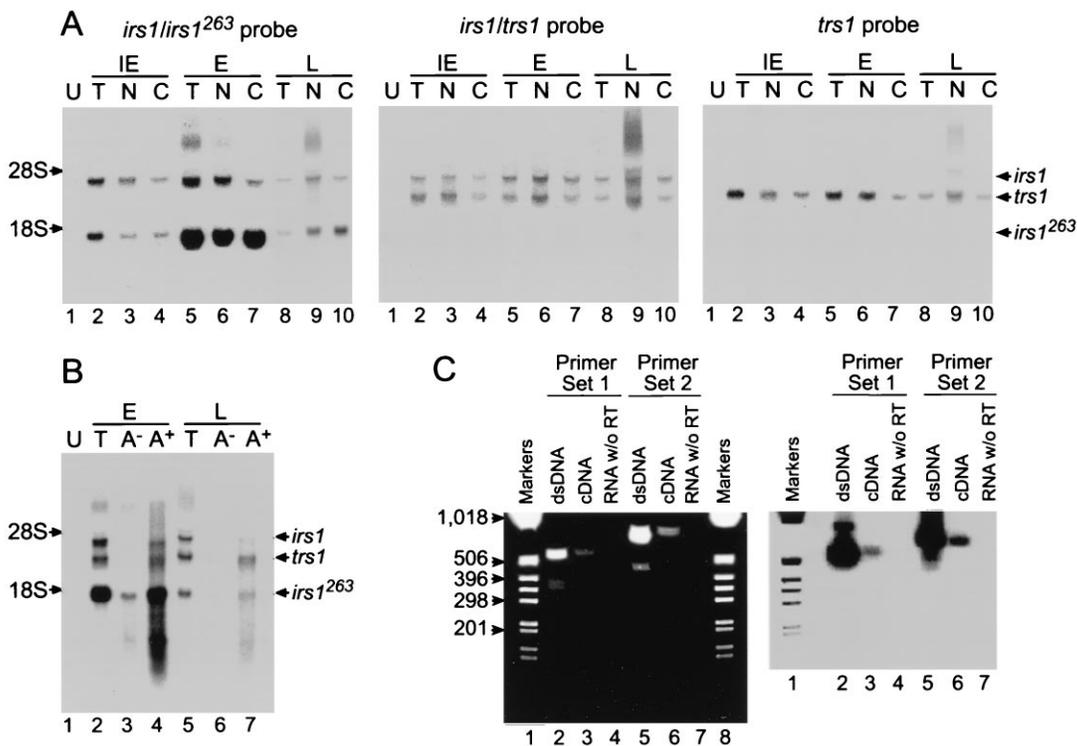

FIG. 2. Analysis of RNAs encoded by the *irs1* and *trs1* genes. (A) Northern blots showing steady-state levels of *irs1*, *irs1²⁶³*, and *trs1* mRNAs. Probe RNAs are those identified in Fig. 1. The source of RNA samples is indicated at the top of the autoradiograms: total-cell RNA from uninfected cells (U) or total-cell RNA from infected cells (T), nuclear RNA (N), or cytoplasmic RNA (C) from cells in the immediate-early (IE), early (E), or late (L) phase of infection. The positions of rRNAs identified by ethidium bromide staining and bands corresponding to viral mRNAs in the autoradiograms are designated. (B) Northern blots of RNAs selected for binding to oligo(dT). Poly(A)⁺ and poly(A)⁻ RNAs are designated A⁺ and A⁻, respectively; all other designations are as for panel A. (C) PCR amplification of reverse-transcribed RNA to compare the sizes of *trs1*-specific cDNAs with that of genomic DNA. PCR amplification employed primers that divided the 3′ end of the *trs1* coding region into two overlapping segments (primer sets 1 and 2). The left panel displays an ethidium bromide-stained agarose gel, and the right panel is the same gel after blotting onto a nylon membrane and probing with a *trs1*-specific antisense riboprobe. The source of DNA amplified is indicated above lanes. cDNA was from cells in the immediate-early phase of infection. w/o RT, control reactions that did not receive reverse transcriptase; dsDNA, double-stranded DNA. The sizes of DNA markers are indicated in base pairs.

tion (Fig. 2A). Their steady-state levels did not appear to change significantly throughout the subsequent stages of the viral replicative cycle and, as previously observed (26), did not accumulate in response to cycloheximide. A new RNA containing *irs1* sequences was detected. This transcript appeared to be about 1,800 nt, it included the 3′ domain of the larger *irs1* mRNA, and we have termed it the *irs1²⁶³* transcript (Fig. 1). Accumulation of the *irs1²⁶³* transcript was highest during the early phase of infection, when it appeared to be present in larger quantities than the *irs1* or *trs1* species (Fig. 2A, left panel). At other stages of infection, it accumulated to levels comparable to those of *irs1* and *trs1* mRNAs. Like the *irs1* and *trs1* RNAs, the *irs1²⁶³* RNA was found in both the nuclear and cytoplasmic fractions of infected cells (Fig. 2A), and it is polyadenylated (Fig. 2B), suggesting that it likely functions as an mRNA.

The *irs1* and *trs1* coding regions of the viral genome were analyzed for presence of potential donor and acceptor splice sites by means of an algorithm devised by Brunak et al. (5). This analysis identified potential donor-acceptor site pairs in the C-terminal domain of *trs1* but none in *irs1*. Since the putative introns in the *trs1* coding region might be too short to generate mRNAs with detectably altered sizes on Northern blots, subdomains of the transcript were analyzed by reverse transcription followed by PCR amplification of the cDNA products (Fig. 2C). The size of amplified cDNA segments was identical to that of the corresponding amplified genomic DNA segments. Thus, we have no evidence for splicing of mRNAs encoded by the *irs1* and *trs1* genes.

Since there was no indication that *irs1*-coded mRNAs are spliced and since the *irs1²⁶³* transcript contained sequences from the C-terminal domain of the larger *irs1* transcript, we considered the possibility that the smaller species was transcribed from a promoter internal to the larger product. Inspection of the *irs1* DNA sequence revealed the presence of a putative TATA motif (ATAAAT) between nt 1684 and 1689 of the *irs1* coding region (Fig. 3). This motif could mark a promoter responsible for the synthesis of the *irs1²⁶³* RNA, and it was not conserved in the *trs1* coding region, which does not produce a smaller transcript. Further, an AUG in a nucleotide sequence context that should efficiently mediate translation initiation (15) was present downstream from the TATA motif in the *irs1* coding region (nt 1750 to 1752) (Fig. 3A). If translation began at this point, located 789 bp upstream from the 3′ end of the *irs1* coding region, a 263-aa polypeptide would be produced, corresponding to the last 263 aa of pIRS1. The *trs1* sequence does not contain an AUG at this position.

To test for the presence of a promoter in the vicinity of the TATA motif located within the *irs1* coding region, a plasmid in which the putative internal *irs1* promoter sequences and translational start site (Fig. 3A) (bp 1 to 1752) were positioned upstream of the luciferase reporter gene was constructed. The internal *irs1* sequence activated the expression of luciferase in uninfected cells and induced expression to a greater extent in HCMV-infected cells (Fig. 3B). Thus, we conclude that an infection-responsive promoter activity is present in the *irs1* coding region that could signal production of an RNA with a size that is similar to that we observe for *irs1²⁶³*.

The apparent size of the *irs1²⁶³* transcript (1,800 nt), together with the presence of a putative TATA motif (sequence positions 1684 to 1689) (Fig. 3A) and initiating AUG (sequence positions 1750 to 1752) (Fig. 3A), suggested that the *irs1²⁶³* mRNA has a 5′ end in the vicinity of sequence position 1715. As an initial test of this hypothesis, we prepared two oligodeoxynucleotide probes (IR263-1 and IR263-2) (Fig. 3A) and predicted that IR263-1 but not IR263-2 should detect the
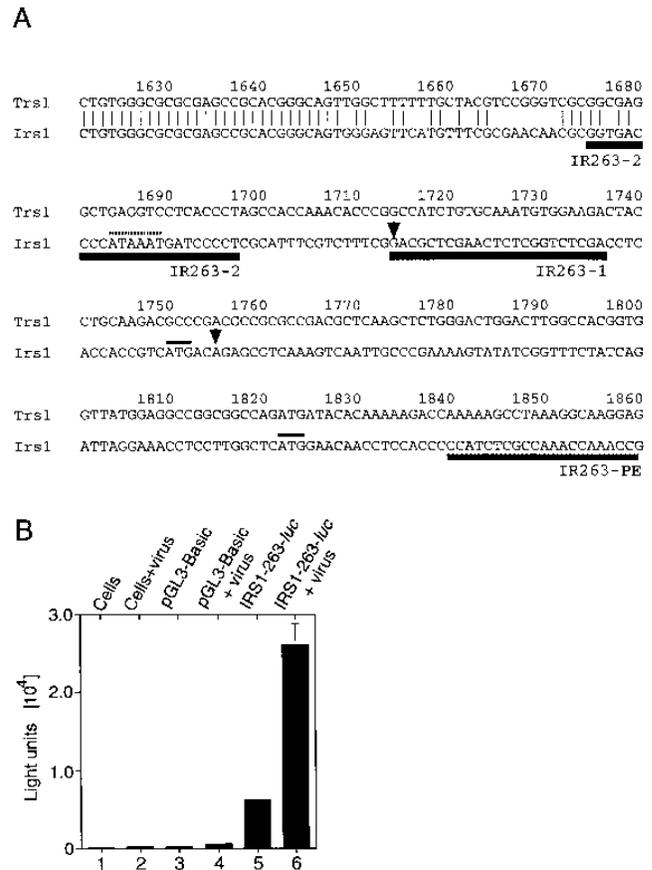

FIG. 3. Identification of a promoter region within the *irs1* coding sequence. (A) Sequence of the 5′ flanking region of the *irs1²⁶³* mRNA in comparison to the equivalent region of the *trs1* gene. The *irs1²⁶³* 5′ end at nt 1714 is designated with an arrowhead. Bars above the sequence identify a putative TATA motif and initiator AUG sequences. Bars below the sequence mark the oligodeoxynucleotide probes used in the Northern blot of Fig. 4A and in primer extension in Fig. 4B. DNA sequence numbers are relative to the start of the *irs1* open reading frame. (B) Transfection assays with the pGL3-IRS1²⁶³-*luc* reporter plasmid or the pGL3-Basic parental plasmid in HFF cells. The average plus standard deviation is presented for three independent assays performed at 48 h after transfection.

irs1²⁶³ mRNA on a Northern blot. This proved to be the case (Fig. 4A). In order to map the location of the 5′ end more precisely, a primer extension analysis was performed using a oligodeoxynucleotide primer located 110 nt downstream from the putative initiating AUG codon. Two extended products were evident in RNA from infected but not uninfected cells (Fig. 4B). One corresponded to a stop site in the primer extension reaction at nt 1755 ± 3, and the other corresponded to nt 1714 ± 3 (Fig. 3A). A 5′ end at position 1714 is consistent with the location of the putative TATA and AUG sequences. The extension product mapping to position 1755 does not include the initiating AUG at positions 1750 to 1752. This extension product might result from a premature termination event in the primer extension reaction. If it represents a bona fide 5′ end of the *irs1²⁶³* mRNA, the resulting mRNA would contain an in-frame initiating AUG at sequence positions 1822 to 1824.

In sum, we have identified a previously undescribed HCMV immediate-early transcript encoded within the *irs1* gene, localizing the promoter responsible for its synthesis and mapping its 5′ end.
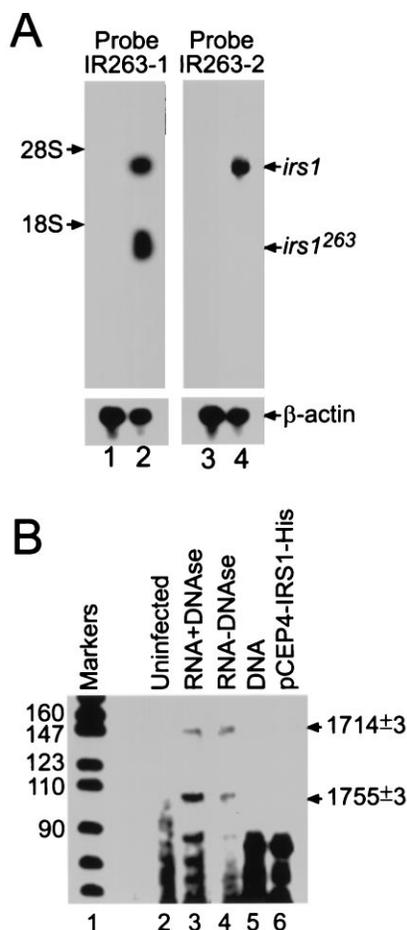
FIG. 4. Localization of the 5′ end of *irs1^263* mRNA. (A) Northern blots using oligodeoxynucleotide probes located near the 5′ end of the *irs1^263* mRNA (locations shown in Fig. 3). The positions of rRNAs (18S and 28S) were determined by staining the gel with ethidium bromide. Bands corresponding to *irs1* and *irs1^263* mRNAs are identified. As a control, membranes were stripped and reprobed with a human β-actin antisense riboprobe synthesized by the T7 polymerase from the pTRI-Human-Actin vector. (B) Primer extension analysis locating the 5′ ends of the *irs1^263* mRNA. The oligodeoxynucleotide primer was located at sequence positions 1840 to 1859 (Fig. 3). The stops identified in the primer extension experiment are numbered relative to bp 1 of the *irs1* open reading frame. The sizes of marker DNAs in base pairs are shown to the left of the autoradiogram.

A new *irs1*-specific protein, pIRS1^263. Monoclonal antibodies that interact with the unique C-terminal regions of pIRS1 and pTRS1, discriminating between the two proteins, were produced. Both proteins were detected by Western blotting at low levels in cell extracts prepared during the immediate-early phase of infection; their levels increased in the early phase and were highest late after infection (Fig. 5A). Similar results were obtained in a time course experiment performed in the absence of drugs (Fig. 5B). The antibody specific to the C-terminal domain of pIRS1 also recognized a shorter polypeptide (about 30 kDa) (Fig. 5A and B). This product corresponds in size to the 263-aa polypeptide that could be produced from the *irs1^263* mRNA if translation began at the AUG located 789 bp upstream from the 3′ end of the *irs1* coding region and the shorter mRNA was colinear with the 3′ end of the *irs1* species. The shorter polypeptide appeared as a doublet in some experiments (Fig. 5A and C) but not in others (Fig. 5B), leading us

to suspect that the faster-migrating component of the doublet results from degradation.

To confirm that the smaller IRS1 protein species could be encoded by an RNA derived from the 3′ domain of the *irs1* mRNA, we performed an in vitro translation experiment (Fig. 5C). A reticulocyte lysate was programmed with a T7 transcript containing the entire *irs1* or *trs1* open reading frame or with a transcript corresponding to the *irs1^263* mRNA. The full-length *irs1* and *trs1* transcripts directed the synthesis of full-length proteins; the smaller IRS1 polypeptide was not produced from the full-length transcript. The shorter *irs1* transcript directed the synthesis of two polypeptides (Fig. 5C) corresponding in size to the doublet detected in late virus-infected cells by the pIRS1-specific monoclonal antibody (Fig. 5A). This result confirms our conclusion that the smaller pIRS1 species are synthesized from an mRNA comprising the 3′ domain of the *irs1* open reading frame. Although we suspect that the *irs1^263*-specific doublet seen in infected cells and in the in vitro translation experiment results from degradation, we cannot rule out the use of two translational initiation sites or posttranslational modification of the protein. Given the presence of two AUGs in sequence contexts that could favor initiation (Fig. 3A) and the fact that the two bands in the doublet correspond in size to the predicted products from the two start sites, it remains possible that two AUGs are utilized for the synthesis of pIRS1^263 polypeptides.

pIRS1 and pTRS1 accumulate in the nucleus and cytoplasm, whereas pIRS1^263 is nuclear. Subcellular localizations were determined by immunofluorescence assay using antibodies to identify pIRS1 and pTRS1 in HCMV-infected HFF cells at each stage of the viral replicative cycle (Fig. 6). Consistent with the Western blot data (Fig. 5), the proteins produce a relatively weak immunofluorescent signal in cells during the immediate-early phase of the infection (protein synthesis was blocked with cycloheximide for 16 h, and the block was reversed for 2 h before analysis), the signal became stronger during the early phase of the infection (cells were infected in the presence of phosphonoacetic acid), and an intense signal was observed during the late phase of infection (no drugs were employed). The cocktail of three monoclonal antibodies (5D2-TRS1C, 9A1-TRS1C, and 9A3-TRS1C) which recognize pTRS1 alone (Fig. 6A to D) and the cocktail of three monoclonal antibodies (1C10-IRS1C, 5H3-IRS1C, and 8B3-IRS1C) which recognize pIRS1 and pIRS1^263 (Fig. 6E to H) revealed identical localization patterns. Both nuclear staining and cytoplasmic staining were evident at immediate-early and early times after infection (Fig. 6B, C, F, and G). Late after infection (86 h), both pIRS1 and pTRS1 exhibited strong cytoplasmic staining with less intense nuclear signal clusters (Fig. 6D and H). To better ascertain whether the apparent nuclear signal observed for pTRS1 late after infection was in fact due to protein in the nucleus, the pTRS1-specific immunofluorescent signal was analyzed by focusing the confocal microscope on three different planes within the same infected HFF cell (Fig. 6I to K). The signal that appeared to be coincident with the nucleus was predominantly localized to the top and bottom optical sections of the cells; little pTRS1 was present in the central section. A substantial portion of the immunofluorescent signal overlapping the nucleus apparently resides in the cytoplasm, and little if any of the signal marks protein present in the nucleus.

Localization of the proteins was also examined in transfected HeLa cells. Cells transfected with pIRS1 (Fig. 7A to C) or pTRS1 (Fig. 7G to I) expression plasmids exhibited strong cytoplasmic staining with little signal evident in the nucleus. Cells transfected with a plasmid expressing pIRS1^263 exhibited
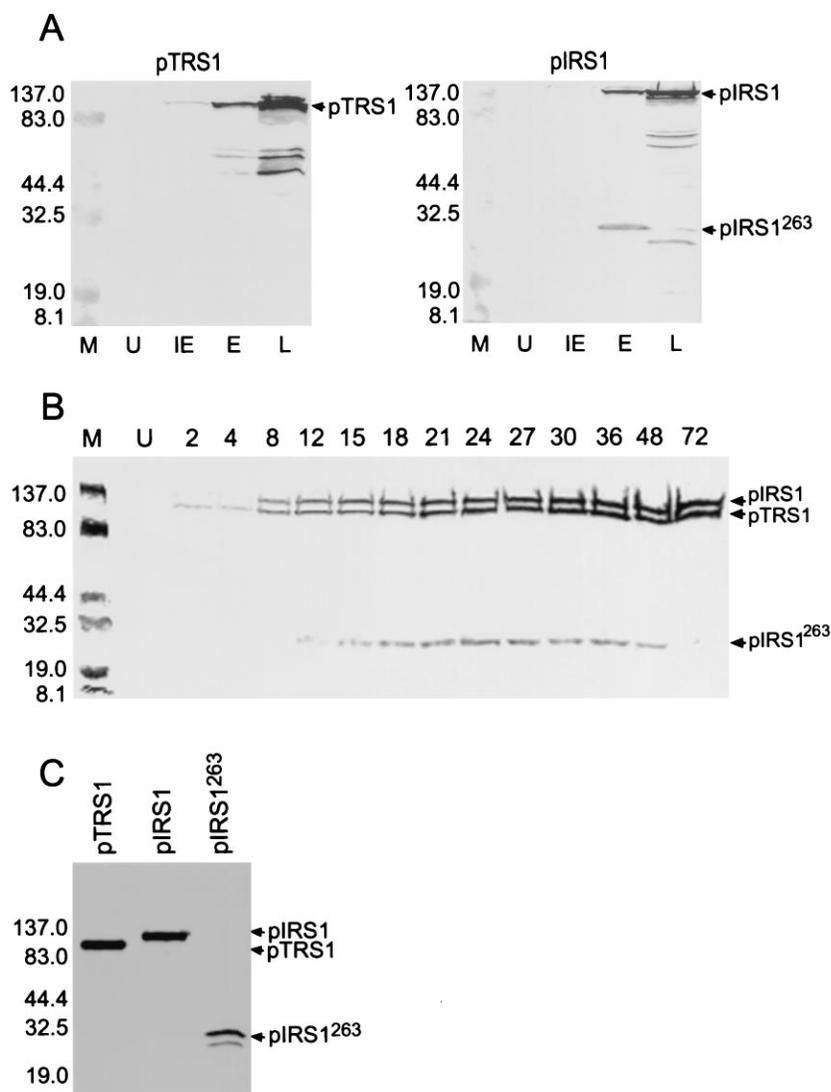
FIG. 5. Analysis of the proteins encoded by the *irs1* and *trs1* open reading frames. (A) Western blot assays in which the *irs1*-specific proteins, pIRS1 and pIRS1[263], and the *trs1*-specific protein, pTRS1, were detected with monoclonal antibodies specific to 200 C-terminal aa of the pIRS1 or pTRS1 protein, respectively. Extracts were prepared from uninfected HFF cells (U) and from HFF cells in the immediate-early (IE), early (E), and late (L) phases of HCMV infection. The sizes of marker proteins (M) in kilodaltons are indicated to the left of the blots. (B) Western blot time course analysis of the accumulation of *irs1*- and *trs1*-specific proteins in the absence of drugs. Times are given at the tops of lanes in hours. (C) Electrophoretic analysis of ^{35}S-labeled pIRS1, pTRS1, and pIRS1[263] proteins prepared by in vitro translation of RNAs synthesized with the T7 RNA polymerase. The sizes of marker proteins in kilodaltons are indicated to the left of the autoradiogram.

a strong nuclear signal as well as a somewhat weaker cytoplasmic signal (Fig. 7D to F).

In sum, pTRS1 is present in both the nucleus and cytoplasm of infected cells during the immediate-early and early phases of infection but accumulates predominantly in the cytoplasm late after infection of HFF cells or within transfected HeLa cells. Since our antibodies recognize both pIRS1 and pIRS1[263], we cannot unambiguously discriminate their localization within infected cells. However, pIRS1 is predominantly cytoplasmic, and pIRS1[263] exhibits a nuclear localization within transfected cells.

Transcriptional regulation by pIRS1, pTRS1, and pIRS1-263. The pIRS1/pTRS1 family of proteins, either alone or in combination with the IE1 and IE2 proteins, had little effect on the activity of the major immediate-early promoter, the promoter used for expression of immediate-early effector proteins in our transfection assays (Fig. 8). IE1 alone had the greatest

effect on the major immediate-early promoter (about threefold), and this effect was substantially muted in the presence of other effector proteins. Therefore, with the possible exception of transfections containing IE1 as the sole effector, we could be confident that effects observed on reporter plasmids resulted from direct effects on the promoters controlling expression of the reporter genes rather than from indirect effects due to changes in the levels of effector proteins.

Next, the effect of pIRS1, pTRS1, and pIRS1[263] was examined on three different promoters: the *irs1*[263] immediate-early promoter, the UL112 early promoter, and the ICP36 late promoter (Fig. 9). Consistent with previously published data for the late promoter (27), we found that pIRS1, pTRS1, and pIRS1[263] failed to activate the UL112 or ICP36 promoter when transfected singly or in combination with each other (Fig. 9, experiments 3 to 5 and 18 to 21). All of the test promoters were activated by IE2 alone (Fig. 9, experiment 7) and a com-
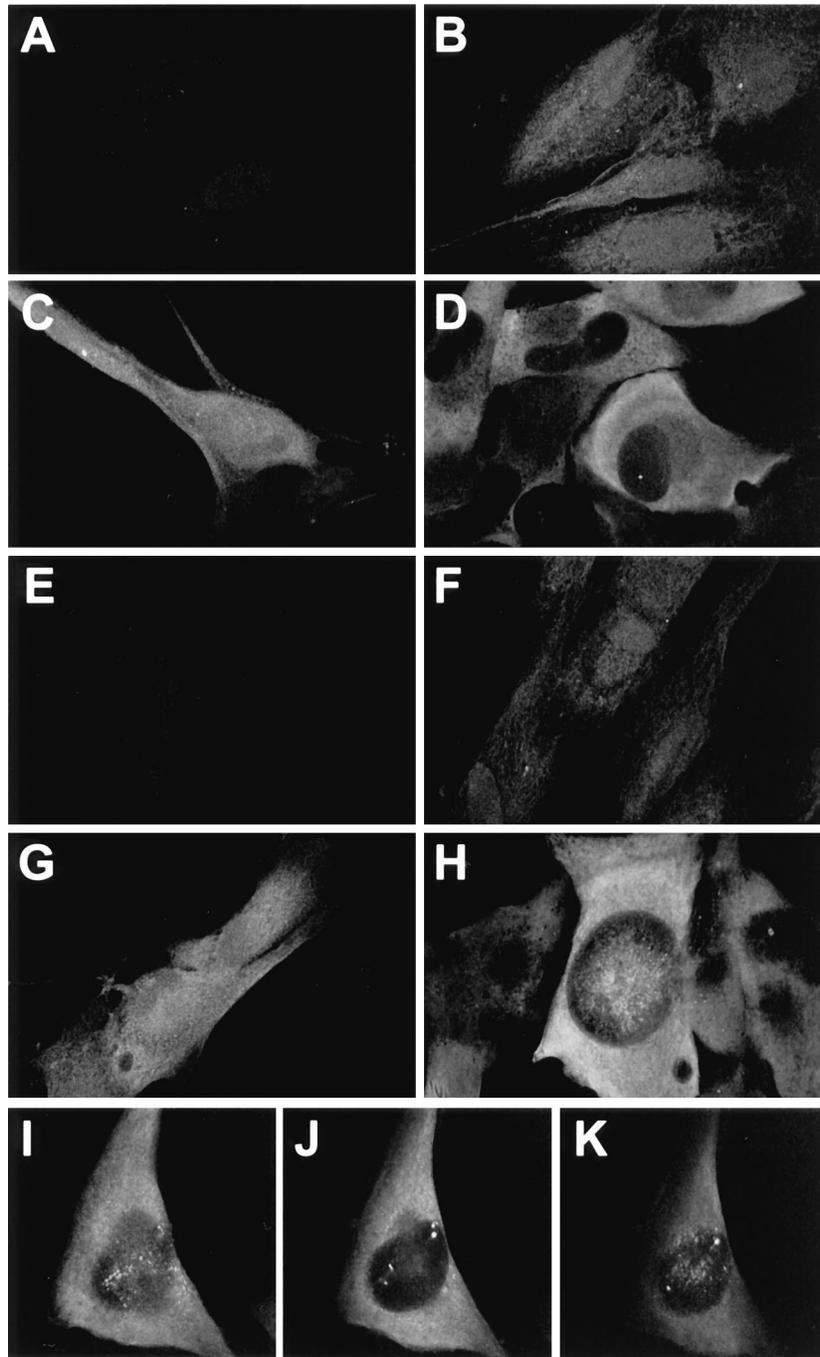
FIG. 6. Immunofluorescent localization of pIRS1 plus pIRS1^{263} (A to D) and pTRS1 (E to K) within HCMV-infected HFF cells. Uninfected cells (A and E) and cells in the immediate-early (B and F), early (C and G), and late (D and H to K) phases of infection are shown. Three optical sections are shown for one late virus-infected cell: bottom (I), middle (J), and top (K).

bination of IE1 and IE2 (Fig. 9, experiment 8), although to different extents. IE1 plus IE2 activated the *irs1*^{263} promoter by a factor of about 17, the UL112 promoter by a factor of about 6, and the ICP36 promoter by a factor of about 3.5. pIRS1 and pTRS1 substantially increased the activity of all promoters in the presence of IE1 and IE2 (Fig. 9, experiments 15 and 16). For example, IE1, IE2, and pTRS1 activated the immediate-early *irs1*^{263} promoter by a factor of 37 (Fig. 9, top panel, experiment 16).

In contrast to pIRS1 and pTRS1, pIRS1^{263} did not function as a coactivator with the IE1 and IE2 proteins (Fig. 9, experiments 11, 14, and 17). Rather, it inhibited expression. For example, IE1, IE2, and pTRS1 cooperated to activate the late ICP36 promoter by a factor of about 10, whereas IE1, IE2, and pIRS1^{263} activated it by a factor of about 2, less than the 3.5-fold activation by IE1 and IE2 alone (Fig. 9, bottom panel, experiments 8, 16, and 17). The antagonistic effect of pIRS1^{263} was confirmed in a titration experiment (Fig. 10). A sixfold
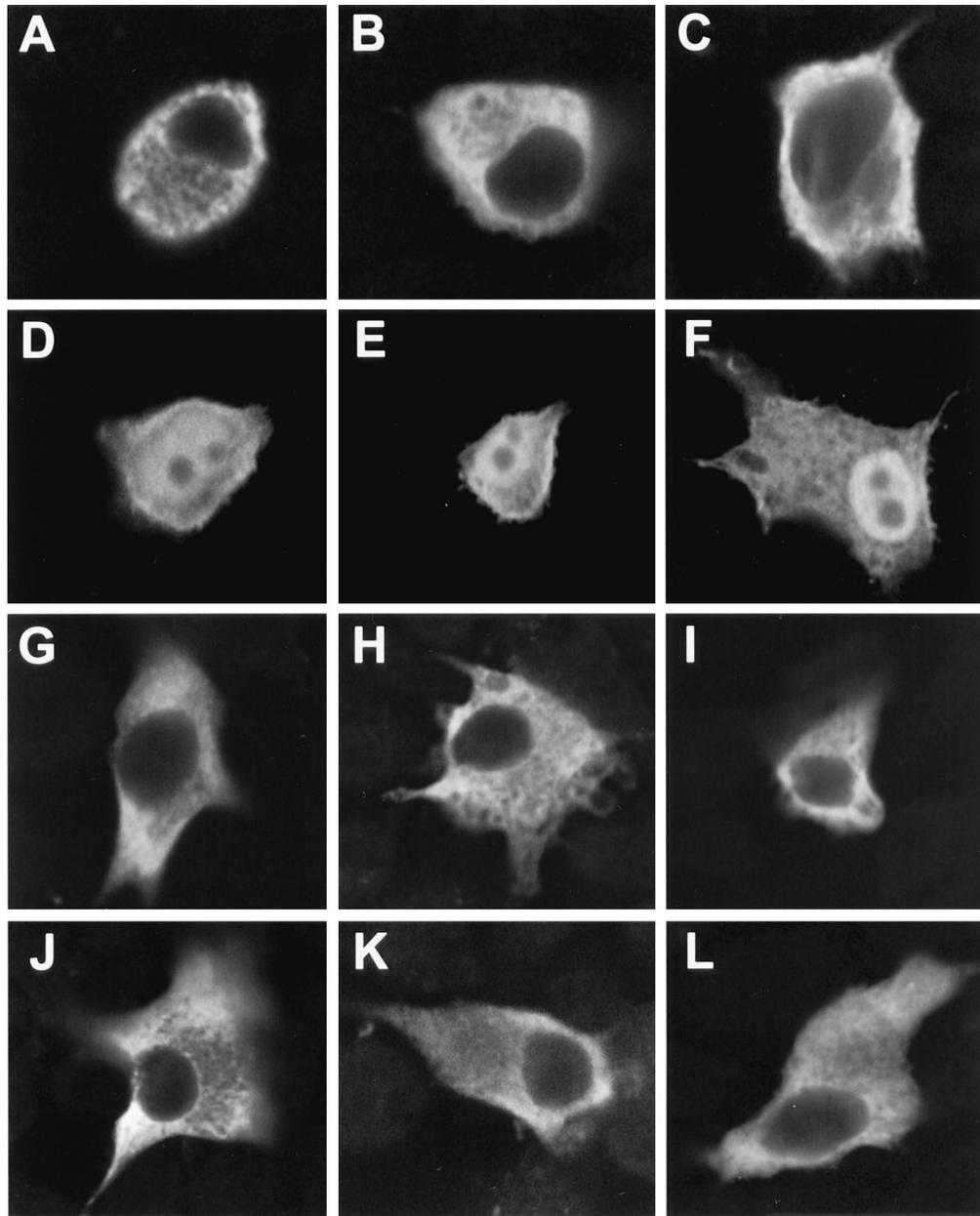
FIG. 7. Immunofluorescent localization of pIRS1 (A to C), pIRS1^{263} (D to F), pTRS1 (G to I), and pTRS1 in the presence of IE1 and IE2 proteins (J to L) in transfected HeLa cells.

molar excess of the pIRS1^{263}-expressing plasmid relative to plasmids expressing IE2 and pTRS1 reduced the level of luciferase activity induced by IE2 and pTRS1 to that induced by IE2 alone. pIRS1^{263} blocked activation by IE2 and pIRS1 equally well (data not shown). This inhibition may explain why the pIRS1 expression plasmid, which encodes pIRS1^{263} as well as pIRS1, generally activated expression in cooperation with IE1 and IE2 proteins somewhat less efficiently than does pTRS1 (Fig. 9, experiments 15 and 16).

Several control experiments were performed to ascertain that the effects of HCMV immediate-early proteins observed in luciferase assays reflected changes in promoter activity. First, we monitored the copy number of a luciferase reporter plasmid in the presence of pTRS1, IE2, or the combination of

pTRS1 and IE2 (Fig. 11A, left panel). The amount of plasmid DNA that could be isolated from cells at 48 h after transfection did not change in response to cotransfection with plasmids expressing the immediate-early proteins. Next, we asked if the level of IE2-specific DNA within transfected cells changed in response to pTRS1, and it did not (Fig. 11A, right panel). Finally, we measured the level of luciferase mRNA (Fig. 11B, left panel). As predicted by the enzymatic assays (Fig. 9), neither pTRS1 nor IE2 alone had a significant effect, whereas the two proteins cooperated to enhance the accumulation of luciferase mRNA from a reporter plasmid containing the HCMV ICP36 promoter by a factor of about 4. rRNA was monitored to control for uniform RNA loading in the electrophoresis experiment (Fig. 11B, right panel). Together, these

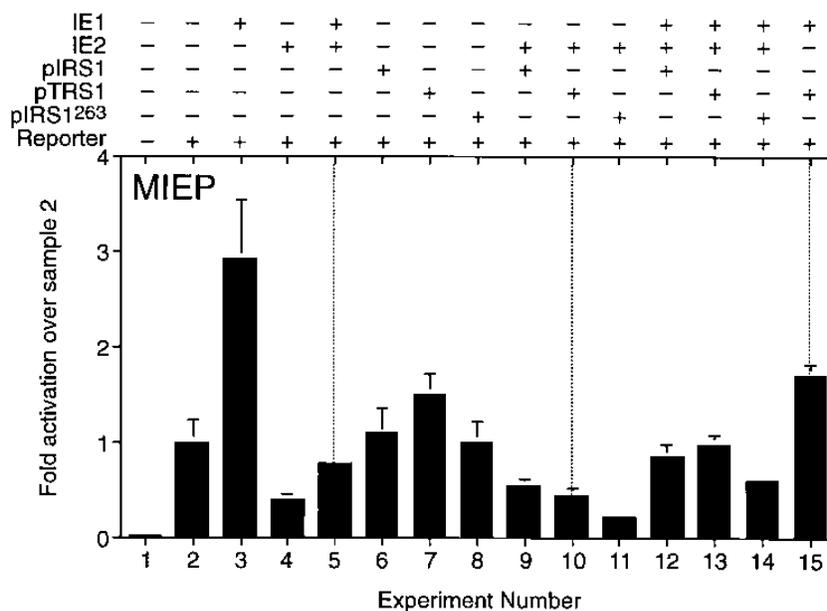|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
IE1	−	−	+	−	+	−	−	−	−	−	−	+	+	+	+
IE2	−	−	−	+	+	−	−	−	+	+	+	+	+	+	+
pIRS1	−	−	−	−	−	+	−	−	+	−	−	+	−	−	−
pTRS1	−	−	−	−	−	−	+	−	−	+	−	−	+	−	+
pIRS1^{263}	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−
Reporter	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+

FIG. 8. Activation of the HCMV major immediate-early promoter (MIEP) within transfected HeLa cells by viral immediate-early proteins. Cells were transfected with the effector plasmids indicated above bars, and luciferase produced by the reporter plasmid controlled by the MIEP was assayed 48 h later. The average plus standard deviation is presented for three different assays.

control experiments argue that the change observed in luciferase activity that we monitored in experiments represented in Fig. 8 to 10 results from changes in reporter mRNA levels.

We conclude that pIRS1 and pTRS1 exhibit little transcriptional activation potential when assayed alone, but each can cooperate with the IE1 and IE2 proteins to activate expression of cotransfected reporter plasmids controlled by viral promoters. In contrast to pIRS1 and pTRS1, pIRS1^{263} antagonizes activation by various combinations of immediate-early gene products.

DISCUSSION

We have characterized the mRNAs and proteins encoded by the HCMV *irs1* and *trs1* genes. RNAs encoded by these genes are expressed in cells infected in the presence of cycloheximide (Fig. 2A), consistent with the earlier characterization of *trs1* as an immediate-early gene (26). The steady-state cytoplasmic levels of *irs1* and *trs1* mRNAs remain constant in cells held in the early phase of infection by phosphoacetic acid and in cells that have proceeded to the late phase of infection (Fig. 2A). Protein products of these genes, pIRS1 and pTRS1, were identified by Western blotting using newly generated monoclonal antibodies (Fig. 5A and B). They were most abundant during the late phase; relatively little pIRS1 and pTRS1 accumulated in the immediate-early phase of infection. The antibodies were also used to localize the proteins in infected and transfected cells (Fig. 6 and 7). Both nuclear and cytoplasmic signals were detected in infected cells during the immediate-early and early phases of infection. The proteins were predominantly if not entirely cytoplasmic during the late phase of infection, and when plasmids expressing pIRS1 and pTRS1 were transfected into cells, the proteins localized to the cytoplasm.

We detected a previously undescribed *irs1* product that we have termed *irs1*^{263} (Fig. 1). The *irs1*^{263} mRNA is evident in Northern blots (Fig. 2A and B) analyzed with a probe corresponding to the C-terminal domain of the *irs1* open reading frame (Fig. 1, probe IRS1C). This RNA is transcribed from a promoter residing within the N-terminal half of the *irs1* open reading frame (Fig. 3A); it has a 5′ end near sequence position 1714 and possibly a second start site near position 1755 (Fig. 4). It is present during the immediate-early and late phases, but it accumulates to the highest levels during the early phase of infection (Fig. 2A). pIRS1^{263} is detected by Western blotting using a pIRS1-specific antibody in extracts of cells prepared during the early or late phase of infection (Fig. 5A and B), it can be translated in vitro from a T7 polymerase-transcribed RNA corresponding to the 3′ domain of the *irs1* open reading frame (Fig. 5C), and it localizes to the nuclei of transfected cells (Fig. 7D to F).

Stasiak and Mocarski (26) previously demonstrated that a combination of pTRS1, IE1, and IE2 was able to activate expression of the late ICP36 promoter. pTRS1 was expressed from its endogenous promoter, leading to an ambiguity in interpretation. The authors suggested that either the three proteins cooperated to activate the ICP36 promoter or the IE1 and IE2 proteins induced expression of pTRS1, which then acted on the ICP36 promoter alone. Recent work from Iskenderian et al. (11) showed that combinations of UL36-38, UL112-113, pIRS1 or pTRS1, and the IE1 and IE2 proteins activate a variety of HCMV promoters at very high efficiency, much more effectively than is observed for IE1 and IE2 alone. Similarly, Kerry et al. (14) showed that combinations of activators including pTRS1 cooperate to activate expression of viral promoters. However, pIRS1 and pTRS1 and other activators were expressed under the control of their natural promoters in these studies, and, again, it is not clear whether the viral proteins cooperated directly to activate target promoters or worked in part indirectly by inducing each other's expression. In our transcriptional analysis, we used the major immediate-early promoter (19, 23) to control expression of IE1, IE2, pIRS1, and pTRS1. This set of proteins had little effect on the activity of the major immediate-early promoter (Fig. 8), but pTRS1, IE1, and IE2 cooperated to activate transcription of the three viral promoters tested, including the ICP36 promoter (Fig. 9). Since IE1 and IE2 do not regulate the promoter
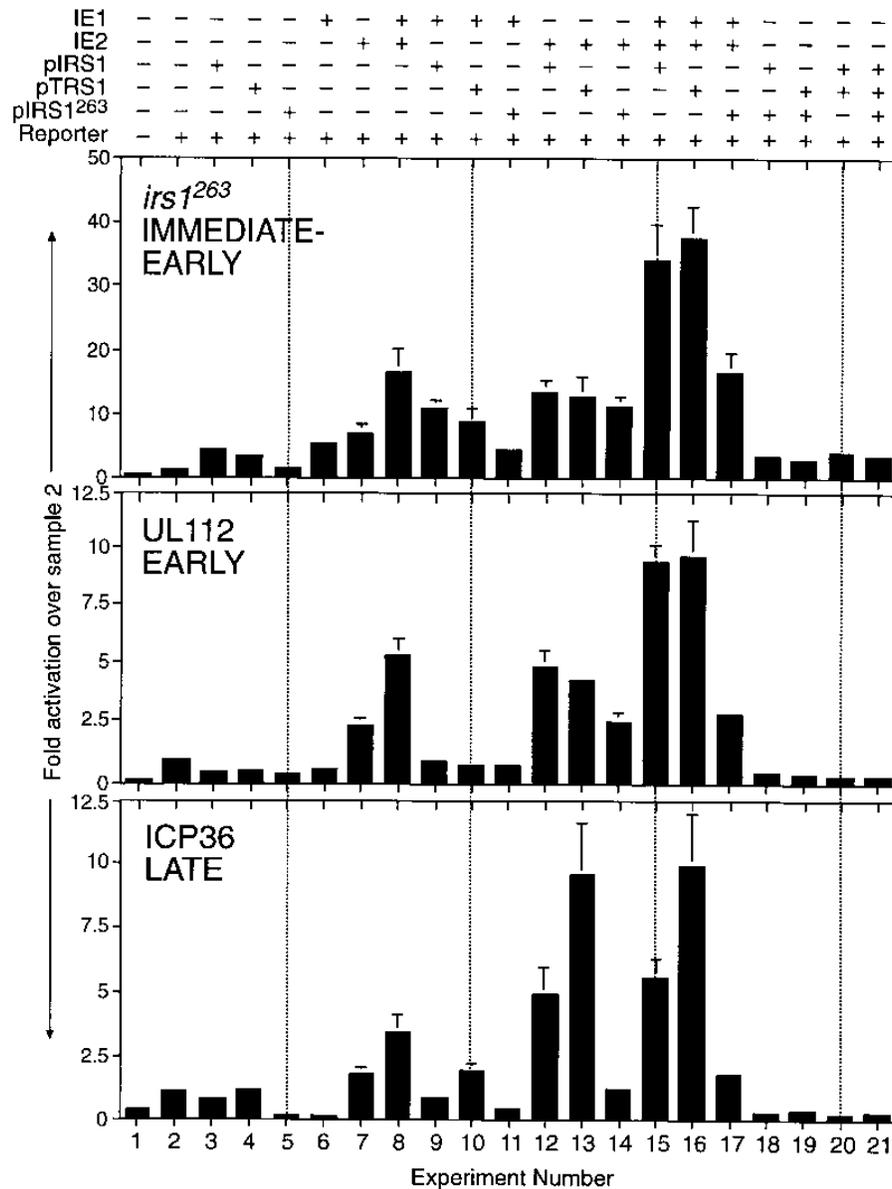
FIG. 9. Activation of an HCMV immediate-early (*irs1*[263]), early (UL112), and late (ICP36) promoter within transfected HeLa cells by viral immediate-early proteins. Cells were transfected with the effector plasmids indicated at the top of the figure, and luciferase produced by the reporter plasmids was assayed 48 h later. The average plus standard deviation is presented for three independent assays.

controlling expression of pTRS1 in this experiment, we can conclude that the three proteins cooperate to influence the activity of the viral promoters in the reporter constructs. Our results further demonstrate that pIRS1 as well as pTRS1 can cooperate with IE1 and IE2 to activate expression of a reporter gene from a variety of HCMV promoters (Fig. 9).

The cytoplasmic localization of pIRS1 and pTRS1 in transfected cells (Fig. 7) leads one to ask whether they enhance expression, at least in part, at the level of translation. This possibility appears unlikely. If pIRS1 and pTRS1 functioned at the level of translation, then they should presumably activate independently of the nuclear IE1 and IE2 proteins. Although pIRS1 and pTRS1 each activated the reporter construct controlled by the *irs1*[263] promoter by a factor of 2 to 3, they failed to induce expression from the UL112 or ICP36 reporter (Fig.

9). Further, if pIRS1 and pTRS1 act at the level of translation, then they should activate each reporter construct to the same extent since the reporters each produce the same mRNA. However, pTRS1 in combination with IE1 and IE2 fails to influence the expression of the major immediate-early promoter (Fig. 8), while either protein cooperates with IE1 and IE2 to substantially increase expression of the ICP36 reporter (Fig. 9). Finally, we have shown directly that pTRS1 cooperates with IE2 to induce the level of luciferase RNA in transfected cells. The level of RNA was enhanced by a factor of about 6 (Fig. 11B), whereas luciferase enzymatic activity was elevated by a factor of approximately 10 (Fig. 9). Therefore, if there is an effect at the level of translation, our experiments argue that it must be minimal (less than twofold).

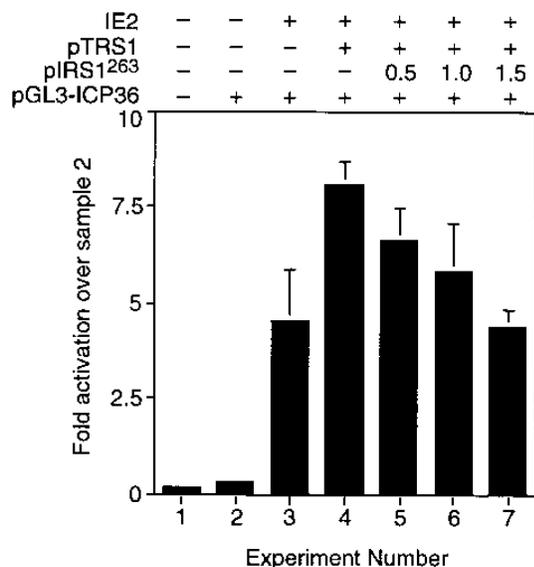As yet, the mechanism by which pIRS1 and pTRS1 cooper-

FIG. 10. Inhibition of luciferase reporter activity by pIRS1[263] within transfected HeLa cells. Cells were transfected with the indicated effector plasmids and a luciferase reporter plasmid, controlled by the ICP36 promoter. Luciferase was assayed 48 h later. Increasing concentrations of the pIRS1[263] expression plasmid (0.5, 1.0, and 1.5 μg/transfection) were used in experiments 5 to 7. The average plus standard deviation is presented for three independent assays.
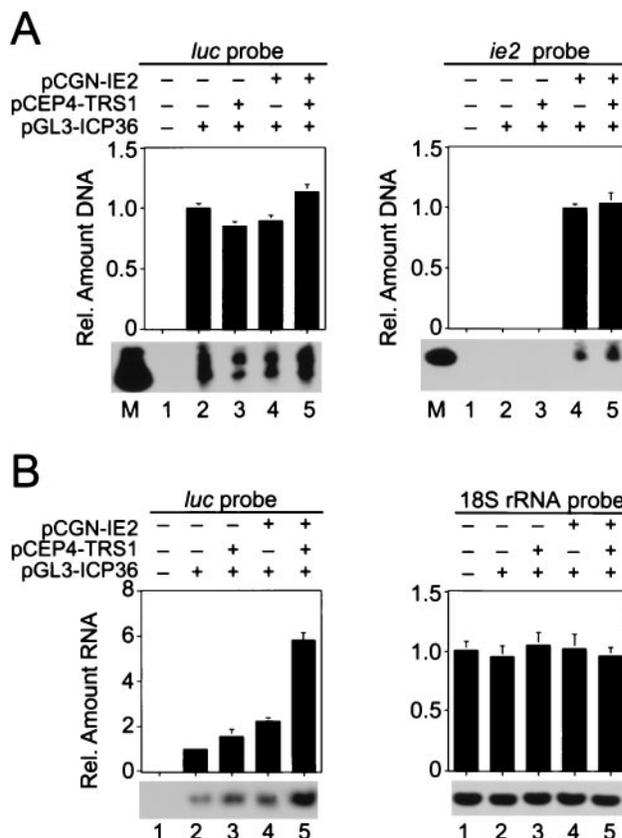

FIG. 11. Analysis of plasmid DNA and reporter RNA produced within transfected HeLa cells. Cells were transfected with the indicated effector plasmids plus the luciferase reporter plasmid controlled by the ICP36 promoter. (A) DNA was prepared at 48 h after transfection and assayed by Southern blotting using either a luciferase-specific riboprobe to monitor the level of reporter plasmid (left panels) or an IE2-specific riboprobe to monitor the level of the IE2-expressing effector plasmid (right panel). The bar graphs show the results of quantifying the radioactivity in the bands representing plasmid DNA by using a PhosphorImager. Standard deviations are derived from three independent experiments. In the autoradiograms, lanes M contain marker plasmid. (B) RNA was prepared at 48 h after transfection and assayed by Northern blotting using a luciferase-specific antisense riboprobe to monitor the steady-state level of reporter RNA (left panels) or an 18S rRNA probe (right panels). Radioactivity was quantified for three independent experiments.

ate with IE1 and IE2 to activate transcription remains unclear. It is possible that the activation that we observe is due to small amounts of pIRS1 and pTRS1 that localize to the nucleus and function directly at the transcription complex. It is also possible that substantial amounts of pIRS1 and pTRS1 are present in the nucleus but are somehow masked so that they cannot be recognized by the monoclonal antibodies. However, this seems unlikely since a cocktail of three different antibodies was used for the analysis. If, as appears likely, pIRS1 and pTRS1 are localized predominantly in the cytoplasm during the late phase of infection (Fig. 6) and in transfected cells (Fig. 7), then they might influence transcription from the cytoplasm. Perhaps these proteins liberate a factor that is normally retained in the cytoplasm, allowing it to move to the nucleus and influence transcription. STAT proteins, which remain in the cytoplasm until they are phosphorylated by Jak kinases and then travel to the nucleus to activate transcription (reviewed in references 8 and 25), provide a precedent for such a regulatory mechanism. Alternatively, pIRS1 and pTRS1 could sequester a factor in the cytoplasm that normally resides in the nucleus and inhibits transcription.

pIRS1[263], which consists of the C-terminal domain of pIRS1, antagonizes transcriptional activation by pIRS1 and pTRS1 (Fig. 9 and 10). In this respect, pIRS1[263] appears to function like the papillomavirus E2TR and E8/E2 proteins. These proteins consist of C-terminal segments of the papillomavirus E2 transactivator protein, and they antagonize transcriptional activation by E2 (reviewed in references 10 and 17). Transcriptional inhibition by the papillomavirus proteins is highly specific for the E2 protein since E2 is a DNA-binding protein and the shorter inhibitory proteins compete for binding to its recognition site. pIRS1[263] differs from the papillomavirus repressor proteins in that its inhibitory activity is not specific to its full-length family members, pIRS1 and pTRS1. It inhibits activation of ICP36 by IE1 and IE2 in the absence of pIRS1 or pTRS1 (Fig. 9). pIRS1[263] accumulates to its highest level during the early phase of HCMV infection (Fig. 2A). Perhaps it

acts to counterbalance activation mediated by a variety of immediate-early proteins. As pIRS1[263] accumulates in response to the same proteins whose activity it antagonizes, it could serve to prevent overexpression of some early or late gene products, optimizing or perhaps slowing the process of viral replication. Whatever its role, the effect of pIRS1[263] on virus growing in cultured cells is at best subtle, because no clear phenotype was evident for a mutant lacking the *irs1* coding region (13).

pIRS1[263] is a predominantly nuclear protein, so it could function by direct interaction with the transcriptional machinery. Although its mechanism of action remains unclear, we are confident that pIRS1[263] does not inhibit expression indirectly due to toxicity because it does not affect the basal activity of the major immediate-early promoter (Fig. 8, experiment 8), its own immediate-early promoter, or the UL112 early promoter (Fig. 9, experiment 5). Work is in progress to elucidate its mode of action.

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