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An Inducible Promoter Mediates Abundant Expression from the Immediate-Early 2 Gene Region of Human Cytomegalovirus at Late Times after Infection

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An abundant late transcript of 1.5 kb originates from the immediate-early 2 (IE-2) gene region of human cytomegalovirus (HCMV) at late times after infection. The transcriptional start site of this RNA was precisely mapped, and the putative promoter region was cloned in front of the CAT gene as reporter. This region, which comprises 78 nucleotides of IE-2 sequence upstream of the determined cap site, was strongly activated by viral superinfection at late times in the replicative cycle. As shown by RNase protection analyses, the authentic transcription start is used. No activation of this late promoter was observed after cotransfection with an expression plasmid containing the HCMV IE-1 and -2 gene region. This result suggests that, compared with early and early late promoters of HCMV, different or additional viral functions are required for the activation of true late promoters.

Gene expression of human cytomegalovirus (HCMV), a member of the beta subgroup of herpesviruses, is regulated in a cascade (10, 11, 21, 36, 37). Three broad phases of gene expression can be distinguished. Immediate-early (IE) genes are expressed in the absence of viral protein synthesis. Their gene products are involved in the switch to the early phase (20, 30). Late genes have been classified into two groups: early-late genes (also called γ1 genes), which are readily detectable prior to and whose expression increases after viral DNA replication; and (true) late genes (also called γ2 genes), which are only detectable after viral DNA replication. Few true late genes have been identified within HCMV (22, 25, 29), and the mechanisms involved in their activation remain to be determined.

One gene locus within HCMV, referred to as the IE-1 and -2 gene region (UL 123-122 [7]), is abundantly transcribed at IE times of infection (18, 33, 38). A strong enhancer element is located immediately upstream of the IE-1 cap site (5, 34). RNAs derived from IE-1 and -2 show a complex splicing pattern (1, 31, 32). Since the first three exons of IE-1 are differentially spliced onto exons of IE-2 (32), it can be shown that the IE-1 enhancer drives expression of both IE-1 and -2 RNAs. For HCMV strain Towne it has been reported that the IE-2 gene region is also heavily transcribed at late times after infection (29, 32). In particular, an abundant late RNA of 1.5 kb originating from within IE-2 in HCMV strain Towne was mapped (31). Using the prototype HCMV strain AD169, we confirmed the late expression and the transcriptional start site of the 1.5-kb IE-2 RNA. We demonstrate that a *trans*-activatable promoter region is composed within a fragment of 78 nucleotides (nt) upstream of the mapped cap site. Although proteins as expressed in transient assays from IE-1 and -2 have been shown to *trans* activate both homologous and heterologous promoters (20, 24, 28), the identified promoter region could not be activated in cotransfection experiments using the major IE-1 and -2 gene region; however, the promoter region strongly responded to viral superinfection.

Northern (RNA) blot experiments were performed to investigate expression of RNAs from the IE-2 gene region of HCMV during the replicative cycle. Human foreskin fibroblasts (HFFs) were infected with HCMV strain AD169 at a multiplicity of infection of 10, and total cellular RNA was harvested at indicated times after infection by the method of Chomczynski and Sacchi (9). After electrophoretic separation of RNA on formaldehyde gels and transfer to nitrocellulose filters as previously described (27), hybridizations were performed by using the *Sma*I-*Xho*I fragment of the IE-2 gene region, labeled by nick translation, as a probe (Fig. 1A). At IE times the major signal observed with the IE-2-specific probe was at a size of 2.2 kb (Fig. 1B, lane 1). A strong reduction in abundance of this 2.2-kb RNA species was found during early times and at late times of infection (Fig. 1B, lanes 2 to 4). At late times additional signals appeared when the IE-2-specific probe was used; the most prominent signal migrated at a size of 1.5 kb (Fig. 1B, lane 4). To test whether expression of this late transcript is dependent on prior viral DNA replication, RNA was harvested at late times after infection in the presence of a 200-μg concentration of phosphonoformic acid, which is known to be a potent inhibitor of herpesvirus DNA polymerases (35). After hybridization with the IE-2-specific probe, no signal could be detected at 1.5 kb (Fig. 1C, lane 2). Thus, the pattern of transcripts originating from IE-2 changes during the time course of infection. Whereas at IE times an RNA of 2.2 kb dominates, this region is also abundantly transcribed at late times after infection. When hybridizations were performed by using probes that are specific for exon 1 or exon 4 of IE-1, the 1.5-kb RNA could not be detected (27), suggesting that expression of this RNA is not driven by the major IE enhancer/promoter.

Primer extension analyses to map the transcriptional start site of the late 1.5-kb RNA were then performed. A 30-mer oligonucleotide primer that was complementary to nt 3218 to 3247 relative to the IE-1 cap site and maps to a position 399 nt downstream of the IE-1 polyadenylation signal was syn-

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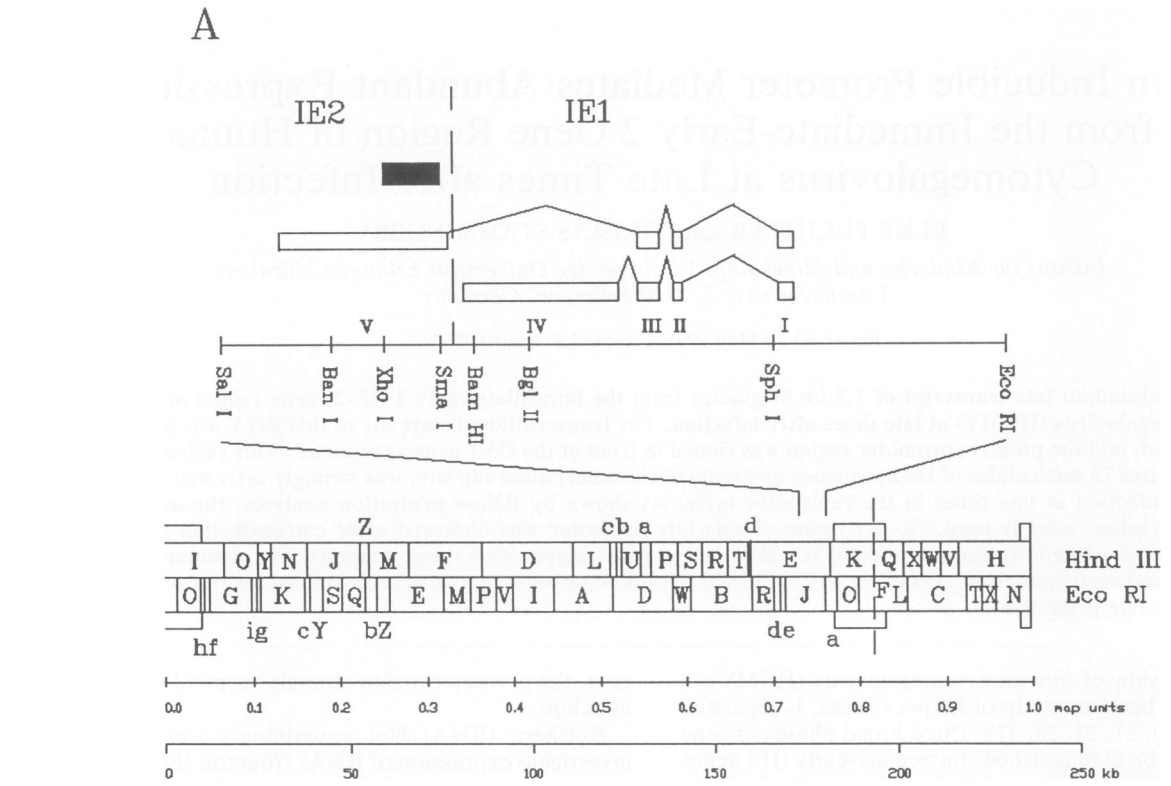

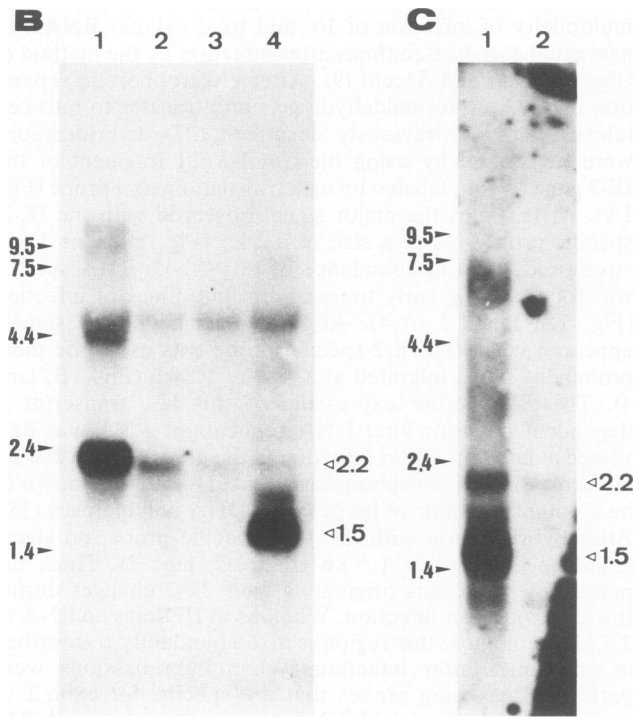

FIG. 1. Northern blot analysis with RNA purified during the HCMV replicative cycle to detect IE-2-specific transcripts. (A) Prototype arrangement of the HCMV AD169 genome and position of the probe used in Northern blot analyses; restriction sites of the IE-1 and -2 gene region of HCMV are shown on top. Open bars, exonic structure (exons I through V) of the two major transcripts from IE-1 and -2 at IE times of infection; solid bar, probe used for detection of IE-2-specific transcripts in Northern blot hybridizations. (B) Lanes 1 to 4, 10 μg of total cellular RNA harvested at 3, 7, 24, and 72 h, respectively, after infection. (C) Lanes 1 and 2, 10 μg of total cellular RNA harvested at 72 h after infection and at 72 h after infection in the presence of phosphonoformic acid, respectively. Sizes (in kilobases) of major transcripts are indicated by open triangles; positions and sizes (in kilobases) of molecular size markers are indicated by arrows.

thesized. After labeling of the oligonucleotide to a high specific activity with [γ-^{32}P]dATP and polynucleotide kinase (GIBCO/BRL, Eggenstein, Germany), primer extension analyses were performed as previously described (3). RNA (40 μg) harvested at 72 h after HCMV infection of HFFs was coprecipitated with 60,000 cpm of the oligonucleotide primer. Hybridization was performed for 12 h at 30°C in a buffer containing 80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, and 1 mM EDTA (pH 8.0). After ethanol precipitation, extension of the primer was achieved by incubation for 90 min at 42°C in the presence of 20 U of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). Extended products were then separated on a 6% denaturing polyacrylamide gel together with a DNA sequencing reaction for size determination. After autoradiography, a prominent signal corresponding to a size of 117 nt could be observed (Fig. 2A). This experiment was performed in triplicate with different RNA preparations, resulting in a reproducible extension pattern. In addition, when RNA that was harvested at IE times of infection was used for primer extension analyses, no signal was visible at this position (data not shown). This argues against a strong stop signal. Together with the finding that the 1.5-kb transcript could not be detected in Northern hybridizations with the IE-1 *Bgl*II-*Bam*HI probe (27), this result identifies the transcriptional

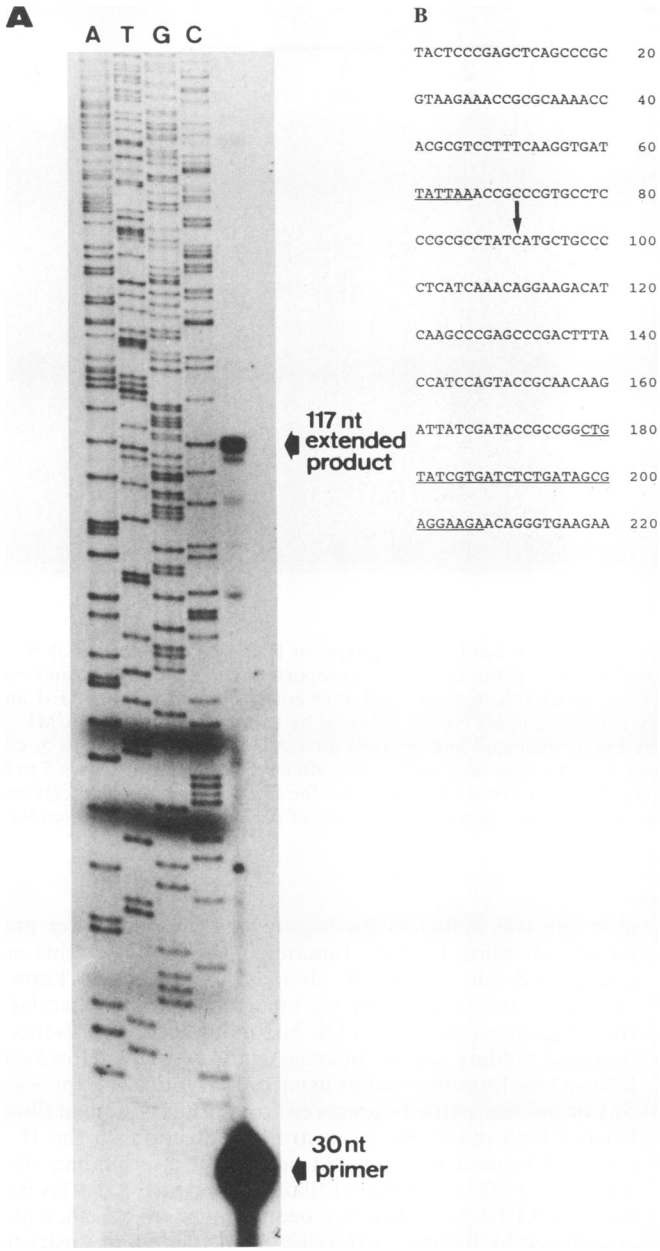

FIG. 2. Primer extension analysis with RNA harvested at late times after infection with HCMV. (A) A 30-mer oligonucleotide which maps to a position 399 nt downstream of the IE-1 cap site was used for hybridization with 40 μg of late RNA. After extension of the primer, fragments were fractionated on 6% polyacrylamide–8 M urea gels along with a DNA sequence ladder for size determination. The sizes (in nucleotides) of the oligonucleotide primer and the extended product are indicated on the right. (B) Nucleotide sequence of the IE-2 region downstream of the IE-1 polyadenylation signal. The initiation site of the 1.5-kb late RNA of IE-2 as determined by primer extension analysis is indicated by an arrow. A potential TATA box sequence and the sequence used as the primer for primer extension are underlined.

start site of the 1.5-kb RNA. It localizes to nt 3131 relative to the IE-1 cap site and is 312 nt downstream of the IE-1 polyadenylation signal. A possible TATA box sequence could be detected 26 nt upstream of the cap site (Fig. 2B). These results confirm the expression pattern and the transcriptional start site of the 1.5-kb IE-2 RNA that were determined with HCMV strain Towne (29).

To test whether the region flanking the mapped 5′ end of the 1.5-kb late IE-2 RNA contains a functional promoter, a 153-nt *Sac*I-*Cla*I fragment comprising a DNA sequence of 78 nt upstream of the cap site was cloned into plasmid puPCAT (26), which was modified by deletion of the IE-1 promoter and by insertion of a synthetic oligonucleotide containing a *Cla*I restriction site (Fig. 3A). Permissive human fibroblasts were then transfected with 20 μg of the resulting plasmid pIE2PCAT as previously described (14), which was followed by superinfection with HCMV strain AD169 at a multiplicity of infection of 5 to 10. Cell extracts were prepared at various times after infection, and chloramphenicol acetyltransferase (CAT) enzyme levels were determined as previously described (14). No promoter activation was detectable after mock infection of transfected cells or during the IE phase of viral gene expression (Fig. 3B, lanes 1, 2, and 6). Low activation could be observed at 24 h after infection (Fig. 3B, lane 3); however, maximum activity was not achieved before the late phase of viral gene expression (Fig. 3B, lanes 4 and 5). Thus, this region strongly responds to viral superinfection at late times of the replicative cycle but not during the early phase of infection, as has been reported for several early promoters of HCMV (6, 28). Cotransfection experiments were performed to see whether the IE-1 and -2 proteins whose expression is driven by the major IE enhancer/promoter are able to *trans*-activate the identified IE-2 promoter region. Plasmid pIE2PCAT (5 μg) was cotransfected with 20 μg of plasmid pRR47 (containing the IE-1 and -2 gene region under control of the major IE enhancer/promoter [27]) into HFFs. As shown in Fig. 3B, lanes 7 and 8, no significant increase in CAT activities was detectable after cotransfection, whereas plasmid pRR47 was able to stimulate expression from several heterologous promoters, including the human immunodeficiency virus long terminal repeat (4; data not shown). This result was obtained in several independent experiments in which various amounts of DNA were used. Therefore, proteins expressed from the IE-1 and -2 gene region in transient assays are not sufficient to stimulate the identified promoter region.

RNase protection experiments were performed in order to test whether activation occurs on the RNA level. Again, HFFs were transfected with plasmid pIE2PCAT, which was followed by superinfection with HCMV or mock infection. RNA was harvested 72 h after transfection and hybridized with a CAT-specific antisense transcript as previously described (26). After RNase digestion and separation of protected fragments on a 6% sequencing gel, a signal, corresponding to the expected size of the CAT fragment (250 nt), could be observed in RNA from HCMV-infected cells; no signal was present in RNA from mock-infected cells (Fig. 4A, lanes 1 and 2). This indicates that activation observed in CAT assays occurs on the level of RNA synthesis. A *Sac*I-*Eco*RI fragment of pIE2PCAT containing about 250 nt of the CAT gene fused to the IE-2 sequences of pIE2PCAT was then subcloned into the BlueScribe Vector (Vector Cloning Systems, San Diego, Calif.) to allow the generation of antisense transcripts (plasmid pIE2PRNP). RNase protection experiments were performed to confirm the start site of transcription within pIE2PCAT after transient expression.

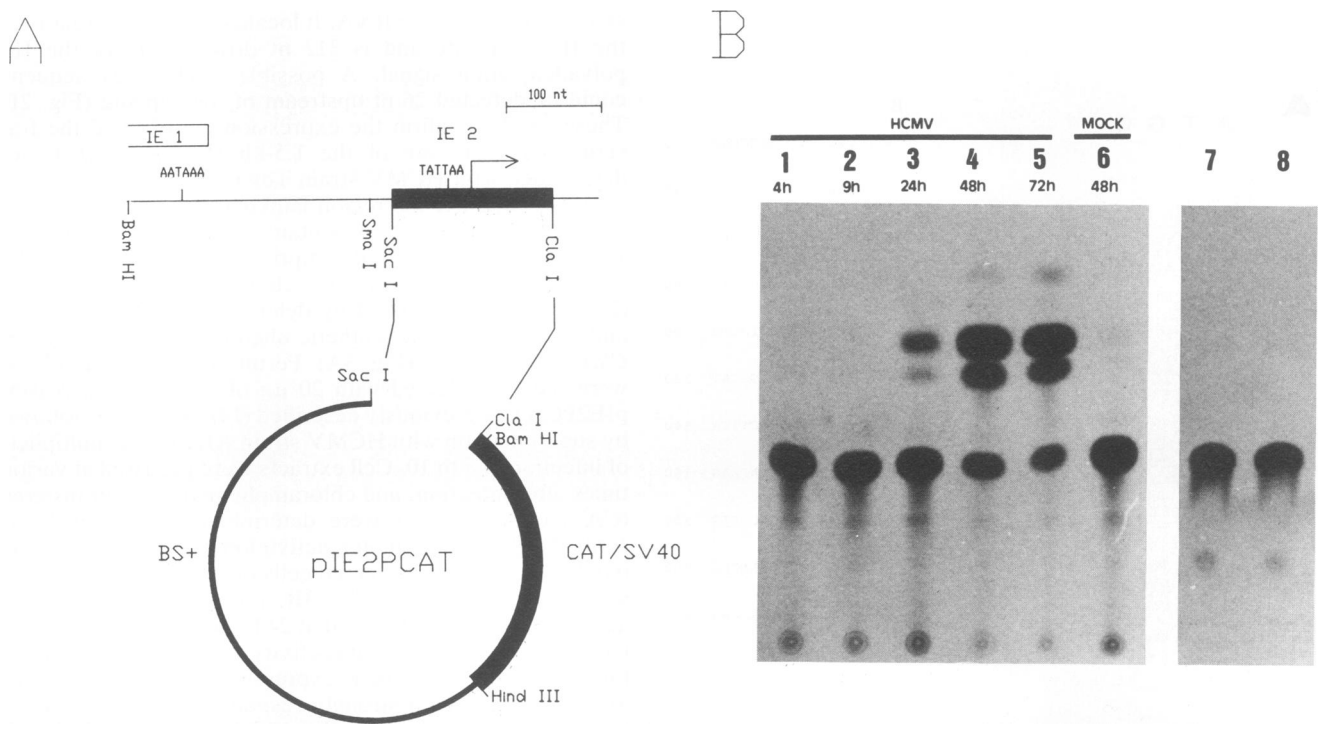

FIG. 3. Cloning of the late IE-2 promoter and activation by viral functions. (A) A *Sac*I-*Cla*I fragment of IE-2 which comprises 78 nt of DNA sequence upstream of the mapped transcription start site was cloned in front of the CAT gene as reporter. The resulting plasmid was designated pIE2PCAT. (B) CAT assay of plasmid pIE2PCAT in HCMV and mock-infected cells and after cotransfection with the IE-1 and -2 gene region. Lanes 1 to 6, plasmid pIE2PCAT (20 μg) was transiently expressed in HFF cells followed by superinfection with HCMV or mock infection. Cell extracts were prepared at various times after infection as indicated and assayed for CAT activity. Lanes 1 to 5, cell lysates prepared at 4, 9, 24, 48, and 72 h after infection with HCMV; lane 6, cell lysate prepared at 48 h after mock infection. Lanes 7 to 8, cotransfection of pIE2PCAT with plasmid pRR47 (containing the IE-1 and -2 gene region under control of the IE-1 enhancer/promoter); lane 7, 5 μg of pIE2PCAT cotransfected with 20 μg of the BlueScribe vector as a negative control; lane 8, 5 μg of pIE2PCAT cotransfected with 20 μg of plasmid pRR47.

RNA (40 μg) of cells that had been transfected with pIE2PCAT followed by superinfection with HCMV or mock infection was hybridized with an antisense transcript derived from pIE2PRNP. After RNase digestion and separation of the resulting fragments, a signal could be observed when RNA of HCMV-infected cells had been used but not with RNA of mock-infected cells (Fig. 4, lanes 2 and 3). This signal had the expected size of about 340 nt. Therefore, the authentic transcription start is used after transient expression of pIE2PCAT which is followed by superinfection.

In summary, this study identifies a promoter located within the IE-2 gene region that mediates abundant transcription at late times of the HCMV replicative cycle. Expression from this promoter within the HCMV genome is strictly dependent on prior viral DNA replication, since no signal could be detected in Northern blot hybridizations when RNA was harvested in the presence of phosphonoformic acid. As was also observed for another true late promoter of HCMV (25), this strict regulation was not reflected in a transient expression assay, in which low levels of activation were seen at early times. Therefore, as with herpes simplex virus late genes (2), the physical environment of the viral genome may be required for proper temporal regulation. However, the activation pattern of these late promoters is clearly different from that observed with early promoters, in which significant activity could be detected as early as 6 h after HCMV infection (28).

A region of 78 nt upstream of the identified transcription start site was sufficient for high-level expression after promoter activation by viral functions. Inspection of this sequence did not reveal any obvious homologies to known eukaryotic transcription factor binding sites other than GC-rich sequences, including a GC box motif which represents a potential binding site for nuclear factor Sp1 (13). However, DNase I footprinting studies using prokaryotically expressed Sp1 or nuclear extracts prepared from primary human fibroblasts which otherwise gave strong protection on the IE-1 enhancer/promoter region did not detect any binding sites within the IE-2 late promoter (data not shown). A TATA box motif, TATTAA, which has been shown to be efficiently recognized by human TFIID (39), is located 26 nt upstream of the cap site. Thus, compared with the complex arrangement of transcription factor binding sites within the major IE enhancer/promoter (15, 16), relatively little sequence information seems to be required for efficient function of an HCMV late promoter.

Activation of the identified promoter depends on viral functions. In contrast to early promoters (20, 28, 32) and early late promoters (12) of HCMV, proteins expressed from IE-1 and -2 after transfection of permissive cells are not sufficient to mediate activation. This was also found when another true late promoter, the promoter of the major capsid gene of HCMV linked to the CAT gene (25), was cotransfected with the IE-1 and -2 expression plasmid (data not shown). Therefore, true late promoters require different or additional viral functions which remain to be defined; how-

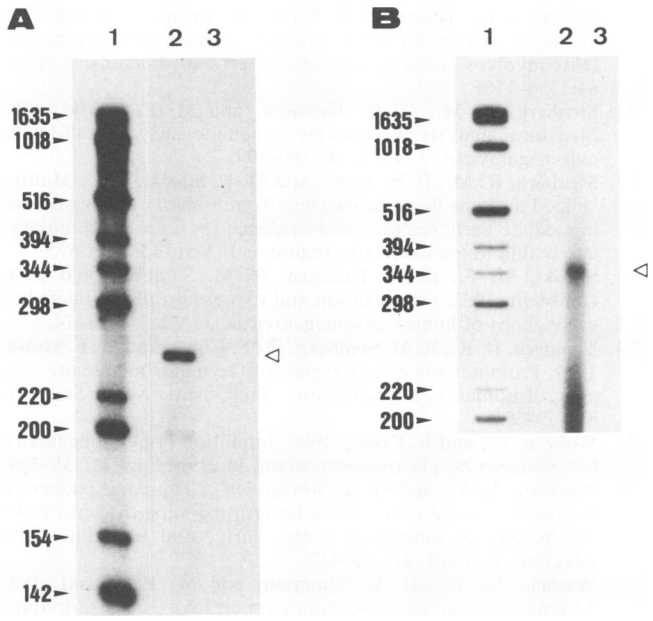

FIG. 4. RNase protection analysis with total cellular RNA harvested from HFF cells after transient expression of pIE2PCAT and then superinfection with HCMV or mock infection. (A) CAT-specific antisense transcript used for hybridization. (B) An antisense transcript derived from plasmid pIE2RNP (which contains the IE-2 sequences of pIE2PCAT fused to about 250 nt of the CAT gene) was used for hybridization. Lane 1, molecular size standard (in kilobases) ladder; lane 2, 40 μg of RNA harvested after transient expression of pIE2PCAT and then superinfection; lane 3, 40 μg of RNA harvested after transient expression of pIE2PCAT and then mock infection. The open triangles indicate the positions of protected fragments; sizes (in nucleotides) of molecular size markers are shown on the left.

ever, the knowledge of the location and sequences of true late promoter elements will certainly help to clarify these mechanisms.

The IE-2 gene region codes at IE times for a protein of 82 kDa apparent molecular mass which is able to *trans*-activate early promoters (20, 30) as well as to repress the major IE enhancer/promoter via a short target sequence near the IE-1 cap site (8, 19, 23). While the whole protein is necessary for *trans*-activation, the carboxy-terminal part seems to be sufficient for IE-1 enhancer/promoter repression (17, 24). The potential protein derived from the IE-2 1.5-kb late RNA would encode this carboxy-terminal part and may therefore be the dominant repressor of IE-1 and -2 enhancer/promoter function at late times of the HCMV replicative cycle.

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