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Guinea Pig Cytomegalovirus Immediate-Early Transcription

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Guinea pig cytomegalovirus (GPCMV) immediate-early (IE) gene expression was analyzed. GPCMV IE RNA was defined as RNA obtained from GPCMV-infected guinea pig cells treated with cycloheximide for 1 h before infection and for 4 h postinfection. Mapping studies showed that GPCMV IE genes are located at several distinct sites on the GPCMV genome. A total of 17 GPCMV IE transcripts were identified, and 9 IE transcripts coded for by three specific regions of the genome (regions I, II, and III) were characterized in detail. A series of recombinant DNA clones were generated to identify the nine IE transcripts. Three of the IE transcripts from region I and three from region III were transcribed in the same direction from overlapping sequences. The 2.0-kilobase (kb) transcript encoded by the *Eco*RI E DNA fragment (region II) was the most abundant IE GPCMV transcript. The cloned GPCMV DNA subfragment that was used to identify the region II *Eco*RI E 2.0-kb transcript did not hybridize to GPCMV early or late RNA, indicating that this transcript is expressed only under IE conditions. Expression of RNAs from the IE genes was also measured during a natural GPCMV infection in the absence of cycloheximide. During the natural infection, the transcripts previously identified under IE cycloheximide block conditions were expressed, and the region II *Eco*RI E 2.0-kb transcript was the most abundant transcript at 1 h postinfection. In addition, a rise and fall in RNA levels was observed during the natural infection, demonstrating the transient nature of expression of these transcripts. We conclude that GPCMV IE gene expression is complex, involving a reasonably large number of genes, and demonstrates some similarities with IE transcription by other CMVs.

Human cytomegalovirus (HCMV) is a significant human pathogen, and its clinical importance has recently become more predominantly recognized. The species specificity of HCMV prevents the study of this virus in animals and necessitates finding an appropriate animal model. Guinea pigs, mice, rats, and hamsters also become infected with their own species-specific cytomegaloviruses (CMVs). The similarities in the pathogenicity of guinea pig cytomegalovirus (GPCMV) and HCMV in their respective hosts are impressive. In both species, acute infection is followed by chronic persistent infection (24), and virus is isolated from the same tissues and organs (1, 23), can cross the placenta causing congenital infection (3, 8, 19, 29), can be transmitted by blood transfusion (2), and can cause labyrinthitis (15, 20, 34, 42, 57) and encephalitis (4, 5). Infection of adult Hartley guinea pigs with salivary gland-passaged virus results in a mononucleosis with splenomegaly, lymph node enlargement, anemia, and circulating lymphocytosis with atypical lymphocytes (18, 37) and resembles HCMV infection in the healthy human adult. GPCMV infection of adult inbred strain 2 guinea pigs causes disseminated disease often accompanied by severe bilateral interstitial pneumonia and susceptibility to superinfection (14) and resembles HCMV infection of the immunocompromised human patient.

Although GPCMV infection in vivo and in vitro can be studied by measuring infectious virus, by histological detection of virus particles, and by detection of antigens with virus-specific antisera, these techniques cannot be used to detect low levels of gene expression or identify which portions of the genome are expressed. To understand GPCMV infections in more detail, it is necessary to characterize the virus at the molecular level and develop molecular reagents that can be used to analyze GPCMV gene expression. We initiated molecular analyses of GPCMV by characterizing GPCMV DNA (26) and preparing recombinant DNA plasmids containing GPCMV DNA fragments spanning most of the genome (16). Cloning GPCMV DNA fragments made it possible to determine the structure of the GPCMV genome (16), generate restriction endonuclease maps of the DNA (16), identify regions of DNA sequence homology with HCMV (26), and use in situ hybridization to detect GPCMV infection in cultured cells (46) and in salivary gland (B. P. Griffith, H. C. Isom, and J. T. Lavallee, unpublished data). GPCMV and HCMV DNAs are similar in size (158×10^6 daltons for GPCMV DNA compared to 150×10^6 daltons for HCMV DNA) and similar in G+C content (54% for GPCMV DNA compared to 57% for HCMV DNA). The GPCMV genome consists of a long unique sequence with terminal repeat sequences but without internal repeat regions and, as such, is less complex structurally than that of HCMV. We have also shown that there is some DNA sequence homology between the two virus DNAs (25, 26); the HCMV Ad169 *Hin*dIII E fragment, a region which contains the major immediate-early (IE) HCMV genes (27) and sequences associated with HCMV transformation (41), contains sequences homologous to GPCMV DNA. When hybridization probes prepared from recombinant plasmids containing fragments of the GPCMV genome were used to detect GPCMV nucleic acids in infected cells in culture by in situ hybridization, a higher percentage of infected cells was observed than seen by histological methods or antigen detection (46). Similarly, when GPCMV DNA fragments were hybridized in situ to salivary gland tissue from GPCMV-infected guinea pigs, the technique was more sensitive than previously used procedures and demonstrated that GPCMV gene expression was detected not only in salivary gland duct cells as had previously been known but

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also in cells outside the duct (Griffith et al., unpublished data).

One approach to understanding the pathogenicity of viruses is to study virus gene expression and regulation of virus gene expression. A great deal is known about regulation of gene expression for many of the herpesviruses including HCMV. GPCMV gene expression and regulation of GPCMV gene expression have not been studied. The purpose of this study was to begin to examine transcription of GPCMV genes, concentrating on IE transcription. We experimentally defined GPCMV IE RNA as RNA obtained from GPCMV-infected guinea pig cells treated with cycloheximide for 1 h before infection and for 4 h postinfection (p.i.). In this study, we identified a series of GPCMV IE transcripts and mapped and determined the direction of transcription for nine of these transcripts. We also examined GPCMV infection at specific time intervals p.i. in the absence of drug treatment.

MATERIALS AND METHODS

Cells and virus. GPCMV (strain 22122, provided by G. D. Hsiung, Veterans Administration Hospital, West Haven, Conn.) was propagated in line 104 clone 1 (104C1) cells (kindly provided by C. H. Evans, National Cancer Institute, Bethesda, Md.). Details of the procedures used for culturing of cells, virus propagation, virus infection, and virus titration were described previously (16, 26).

Recombinant plasmids. The plasmids containing fragments of GPCMV DNA produced by *Hin*dIII or *Eco*RI restriction endonuclease digestion were constructed as previously described (16). Plasmids containing small subfragments of GPCMV *Hin*dIII and *Eco*RI DNA fragments were also constructed as described previously (16). DNA cleaved by restriction endonuclease digestion with *Hin*dIII, *Eco*RI, *Bam*HI, *Pst*I, *Sal*I, *Xba*I, and *Sac*I was purified and cloned into the multicloning sites of pGEM-1 or pGEM-2 (Promega Biotec, Madison, Wis.). Plasmid DNAs were isolated and purified by previously described methods (16), and the identity was verified by digestion with the appropriate restriction endonuclease followed by electrophoresis on 1.0 to 1.5% agarose gels.

Construction of restriction endonuclease cleavage maps. Cleavage maps were determined by digestion of GPCMV DNA fragments with multiple restriction endonucleases and/or hybridization with overlapping fragments.

RNA preparation. Cytoplasmic RNAs were isolated and purified from mock- and GPCMV-infected cells by a detergent lysate method as described previously (54). 104C1 cells at 90% confluence were infected at a multiplicity of infection of 10 to 20 PFU per cell and harvested at various times p.i. depending on whether IE, early, or late RNA was prepared. GPCMV IE RNA was defined experimentally as the RNA isolated from GPCMV-infected cells treated with cycloheximide (200 μg/ml) for 1 h before infection and for 4 h p.i. Treatment with cycloheximide at 200 μg/ml blocked cellular and virus protein synthesis in uninfected and GPCMV-infected guinea pig cells. GPCMV early RNA was defined as the RNA isolated from cells treated with phosphonoacetic acid (200 μg/ml) during infection and harvested at 20 to 24 h p.i., and GPCMV late RNA was defined as the RNA isolated from cells at 48 to 72 h p.i. For experiments in which the time course of GPCMV RNA expression was measured in the absence of drug treatment, RNAs were isolated from GPCMV-infected cells at the indicated time points p.i. To prepare RNA, the cells were trypsinized at the time of harvest and washed in phosphate-buffered saline. The cell pellets were then suspended in 0.01 M Tris buffer (pH 7.5) containing 0.5% Nonidet P-40. Nuclei were pelleted by centrifugation, and the supernatant containing the cytoplasmic fraction was brought to a final concentration of 0.1% sodium dodecyl sulfate. RNA was then purified by phenol-chloroform extraction and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. To prepare poly(A)$^+$ RNA, we subjected cellular RNA to chromatography on oligo(dT)-cellulose (Collaborative Research, Inc., Lexington, Mass.). Poly(A)$^+$ RNA (1 to 2 μg) was loaded on each lane for Northern (RNA) analysis.

Synthesis of cDNA and Southern blot analysis. Complementary DNA was synthesized from poly(A)$^+$ RNA templates with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) by a previously published method (27). Southern blot hybridization of cDNA probe to immobilized virus DNA or cloned DNA fragments was done at 46°C overnight. Washing procedures and autoradiography were done as previously described (16).

Northern blot hybridization. Poly(A)$^+$ RNAs were denatured at 60°C for 5 min and fractionated by electrophoresis in 1.2% denaturing agarose gels in MOPS buffer (0.2 M morpholinepropanesulfonic acid [pH 7.0], 50 mM sodium acetate, 1 mM EDTA) containing 2.2 M formaldehyde. RNAs were transferred to nitrocellulose paper as previously described (6). The RNA blots were hybridized with nick-translation-labeled plasmid GPCMV DNA or with riboprobes generated from the SP6 or T7 promoter of the corresponding clones of pGEM-1 or -2 as described in the instructions from the manufacturer. The hybridization was performed in 50% formamide buffer at 50°C overnight for DNA probes or at 60 to 65°C for 4 to 6 h for riboprobes, using 3×10^6 cpm of DNA probe or riboprobe per ml of buffer. The blots were then washed three times at 60°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and once with 2× SSC, once at room temperature with 0.1× SSC–0.1% sodium dodecyl sulfate, and once with 2× SSC. The blots hybridized with riboprobes were washed, treated with 45 U of RNase A in 100 ml of 2× SSC at room temperature with shaking for 30 min, and subsequently rinsed in 2× SSC for 15 min. The blots were air dried and subjected to autoradiography.

Preparation of in vivo-labeled RNA probes. 104C1 cells were fed with medium deficient in phosphorus for 4 h and then labeled with $^{32}P_i$ (50 μCi/ml; Amersham Corp., Arlington Heights, Ill.) for 1 h before infection and for 4 h p.i. Cells were infected with GPCMV under IE conditions described above. Cytoplasmic RNA was isolated from the labeled cells and purified as described above.

DNA dot-blot hybridization. DNA dot-blot hybridization was done as previously described (9, 22). The plasmid DNAs of GPCMV were denatured in 0.1 N NaOH in boiling water for 5 min and neutralized with HCl on ice. DNA samples (5 μg in 500 μl of 6× SSC) were blotted to nitrocellulose filters with a Minifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.) as described by the manufacturer. The blots were hybridized with in vivo-labeled RNA probes. RNA probes were degraded into nucleotides several hundred bases long with 0.2 N NaOH on ice for 30 min. DNAs on the blot were hybridized with in vivo-labeled RNA at 65°C for 4 h in 2× TESS (0.01 M TES [*N*-tri(hydromethyl)methyl-2-aminoethanesulfonic acid] [pH 7.4], 0.3 M NaCl, 0.01 M EDTA, 0.2% sodium dodecyl sulfate) buffer containing 0.05% polyvinylpyrrolidone and 0.05% Ficoll. The washing procedures were done as described previously (22). After

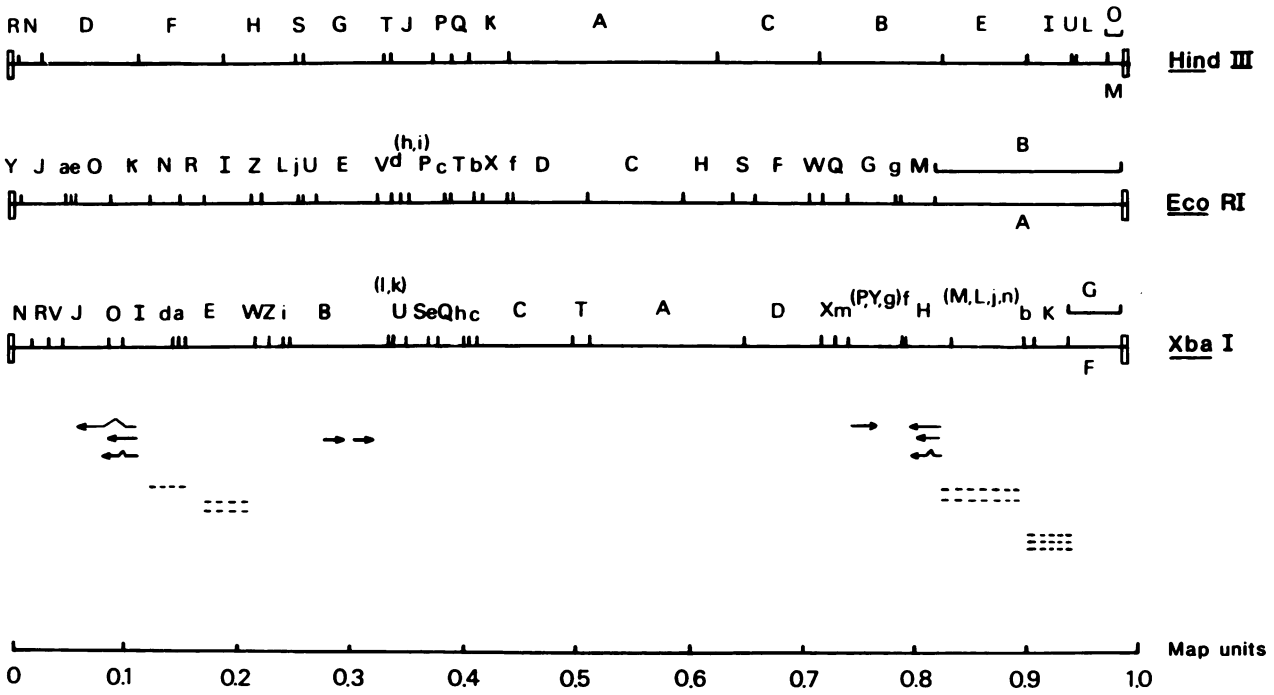

FIG. 1. Schematic diagram of the GPCMV genome with restriction sites for *Hin*dIII, *Eco*RI, and *Xba*I. Parentheses indicate that the order of fragments in this region is unknown. Open rectangles at the termini indicate regions containing sequence homology. The lines with and without arrows indicate the map positions of the IE transcripts. This figure is a modification of the original GPCMV restriction endonuclease map (15).

being washed, the blots were treated with 45 U of RNase A in 100 ml of 2× SSC at room temperature with shaking for 30 min and subsequently rinsed in 2× SSC for 15 min. The blots were air dried and subjected to autoradiography.

RNA dot-blot hybridization. The cytoplasmic RNA dot-blot procedure was done as described previously (56). RNAs were suspended in 1× TE (10 mM Tris [pH 8.0], 1 mM EDTA) and denatured in 0.6 volume of 15× SSC and 0.4 volume of 37% formaldehyde at 60°C for 15 min and placed on ice immediately. The RNA samples (5 μg/150 μl of 15× SSC) were spotted on nitrocellulose paper as described in the DNA dot-blot procedure above. The blots were hybridized with nick-translation-labeled DNA probes, air dried, and subjected to autoradiography. The hybridization and washing were done in the same conditions as described above for Northern blot hybridization.

RESULTS

Temporal regulation of GPCMV transcription. Experiments were done to determine the regions of the GPCMV genome that encode the IE, early, and late transcripts. GPCMV IE, early, and late RNAs are defined in the Materials and Methods section. cDNA probes were synthesized from IE, early, and late poly(A)$^+$ RNAs and hybridized to cloned GPCMV *Hin*dIII and *Eco*RI DNA fragments that span the GPCMV genome (25) (Fig. 1 and 2). The resulting Southern blots were subjected to autoradiography. Scanning the autoradiograms showed that more than 70% of the IE transcripts were derived from sequences contained within the *Hin*dIII D, *Hin*dIII G (*Eco*RI E), and *Hin*dIII B fragments. Early RNAs were transcribed from 16 of 18 cloned fragments, but 35% of the early RNAs were derived from the *Hin*dIII N and L fragments. Late RNAs were transcribed from recombinant DNAs representing 99% of the virus genome.

Analysis of GPCMV IE RNAs encoded by region I. Three GPCMV DNA fragments containing sequences that hybridize to IE RNA were designated regions I (*Hin*dIII-D), II (*Eco*RI-E), and III (*Hin*dIII-B), respectively, and were analyzed in detail. To map the GPCMV IE transcripts within the three regions, detailed restriction endonuclease maps were generated and subclones containing smaller GPCMV DNA fragments were prepared. The *Hin*dIII, *Xba*I, *Eco*RI, *Bam*HI, and *Pst*I restriction endonuclease sites for region I were determined (Fig. 3A).

Northern blot analysis was done to map the IE transcripts encoded by region I. Poly(A)$^+$ cytoplasmic IE RNA extracted from GPCMV-infected cells and immobilized on nitrocellulose was hybridized to nick-translated GPCMV DNA fragments. Initial experiments, which were done with the *Eco*RI J, a, e, O, and K DNA fragments as probes, demonstrated that only DNA sequences from the right end of the *Hin*dIII D fragment, that is, from the *Eco*RI O and K fragments, hybridized to GPCMV IE RNA (data not shown). Northern analysis was then done with smaller GPCMV DNA fragments contained within the *Eco*RI O and K DNA fragments (Fig. 3B). Analysis of autoradiograms of the blots showed that (i) three different size classes of RNAs (3.9, 3.3, and 2.0 kilobases [kb]) were transcribed, (ii) the three RNAs were transcribed from overlapping regions of the genome and initiated near the right end of the *Hin*dIII D DNA fragment, (iii) the 3.9- and 2.0-kb transcripts were spliced, and (iv) the three transcripts were expressed at reasonably equal levels.

To determine whether the RNAs were transcribed from

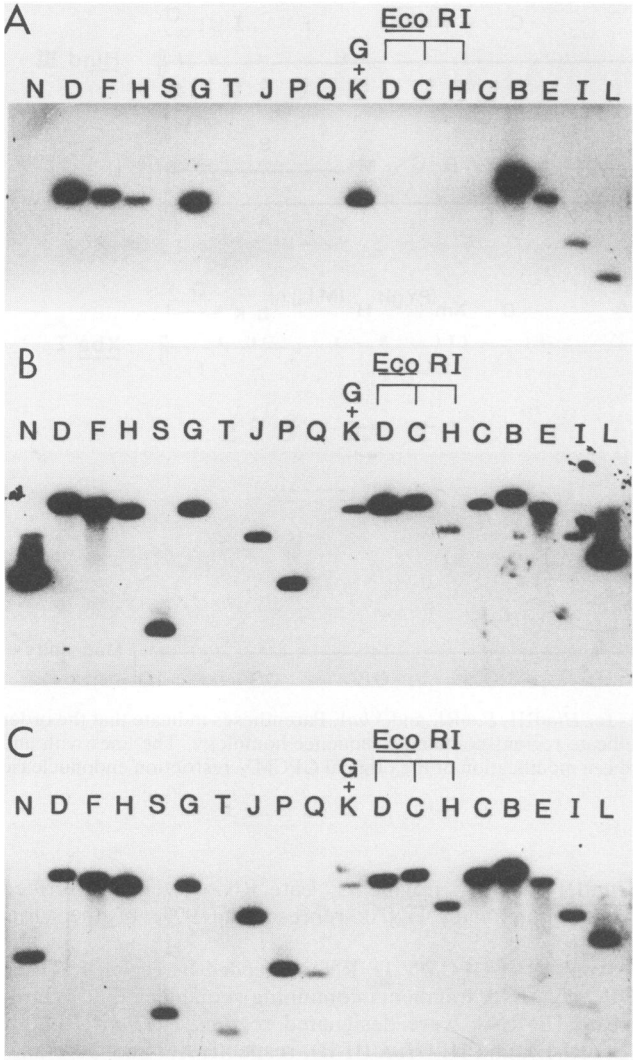

FIG. 2. Hybridization of labeled cDNA probes from IE, early, and late RNAs to GPCMV DNA fragments from recombinant plasmids. GPCMV *Hin*dIII and *Eco*RI fragments were arranged according to their map positions. Recombinant plasmids were cleaved with respective restriction endonucleases and separated on a 0.5% agarose gel. The DNA bands were transferred to nitrocellulose filters and hybridized to ^{32}P-labeled cDNA synthesized from poly(A)$^+$ GPCMV IE (A), early (B), or late (C) RNAs.

the same or opposite DNA strands, RNA-RNA hybridization was done with strand-specific probes. Several smaller region I DNA fragments (EXa, XOPB, and XH) were subcloned into the pGEM-1 vector containing SP6 and T7 promoters, and two cRNAs were transcribed in vitro from each fragment, one initiating from the SP6 promoter and one from the T7 promoter. Analysis of autoradiograms for the RNA-RNA hybridizations revealed that all three region I transcripts were transcribed in the same direction (Fig. 3C). It appears that the multiple overlapping transcripts may initiate from the same promoter and that different-sized transcripts are generated by differential splicing, but mapping in even greater detail is necessary before this conclusion can be verified.

Analysis of GPCMV IE RNAs encoded by region II. The same strategy used to study region I was also used to analyze IE transcription from regions II and III. To map IE transcription from region II, we determined the *Sac*I, *Sal*I, and *Pst*I restriction endonuclease sites within the *Eco*RI E fragment (Fig. 4A). Northern blot analysis was done to map the IE transcripts encoded by region II as had been done for region I (Fig. 4B). Initial experiments which were done with *Eco*RI-*Sac*I (ES′), *Sac*I-*Sac*I (S′S′a, S′S′b), and *Sac*I-*Eco*RI (S′E) fragments spanning the *Eco*RI E fragment showed that only the S′S′a and S′S′b DNA fragments hybridized to GPCMV IE RNA. When hybridization was done with smaller DNA fragments contained within the S′S′a fragment, it was apparent that the *Sal*I-*Sac*I (SS′) DNA fragment at the right end of the S′S′a fragment did not contain DNA sequences that encode GPCMV IE RNA. The results showed that the 3.8-kb transcript is transcribed from the left half of the *Eco*RI E fragment, while the 2.0-kb transcript is transcribed from the right half and that they do not overlap. Also, the 2.0-kb transcript was present in significantly higher levels than the 3.8-kb transcript; in fact, of all the GPCMV IE transcripts that we studied, the region II 2.0-kb transcript was one of the most abundantly expressed. The significance of the 2.0-kb transcript is discussed in more detail below.

To determine whether the RNAs were transcribed from the same or opposite DNA strands, we did RNA-RNA hybridization using strand-specific probes transcribed from subclones containing the PS and SP DNA fragments. The PS DNA fragment was subcloned into the pGEM-2 vector. The 2.0- and 3.8-kb transcripts were transcribed in the same direction (Fig. 4C).

Analysis of GPCMV IE RNAs encoded by region III. To analyze IE transcription from region III, we determined the *Hin*dIII, *Eco*RI, *Bam*HI, *Pst*I, and *Sal*I restriction endonuclease sites for region III (Fig. 5A). When Northern blot analysis was done with the large *Hin*dIII B fragment, at least three intense RNA bands were detected (Fig. 5B). Further analysis with the *Eco*RI M, g, and G fragments as probes showed that four transcripts were encoded by region III, one from the *Eco*RI G DNA fragment and three from *Eco*RI M. Northern analyses with smaller DNA fragments contained within the *Eco*RI G and M DNA fragments showed that (i) a single 2.9-kb transcript hybridized to DNA sequences at the left end of the *Eco*RI G fragment, (ii) three overlapping transcripts, 3.6, 2.7, and 1.9 kb in size, hybridized to sequences at the right end of the *Eco*RI M fragment, and (iii) the 1.9-kb fragment is spliced. The subcloned EH DNA fragment hybridizes weakly to the 3.6-, 2.7-, and 1.9-kb transcripts. Hybridization of EH DNA to the 1.9-kb fragment was difficult to detect except on overexposed autoradiographs. The 2.7- and 2.9-kb transcripts were the most abundantly transcribed region III transcripts.

A series of small region III DNA fragments (EB, SP, PB, BE, EH) were subcloned into the pGEM-1 or pGEM-2 or both vectors to use RNA-RNA hybridization to determine the direction of transcription of the four region III transcripts (Fig. 5C). Analysis of autoradiograms for the RNA-RNA hybridizations showed that the three overlapping transcripts were transcribed from one strand of DNA and that the 2.9-kb transcript was transcribed from the other.

Expression of GPCMV RNAs in the absence of cycloheximide. We were interested in determining whether the same GPCMV genes that were transcribed in the presence of cycloheximide were also transcribed at early times during a natural productive in vitro infection. To do these studies, we radioactively labeled GPCMV-infected cells with $^{32}P_i$, extracted radioactively labeled RNA from the cells at various times after infection, and hybridized the labeled RNA to

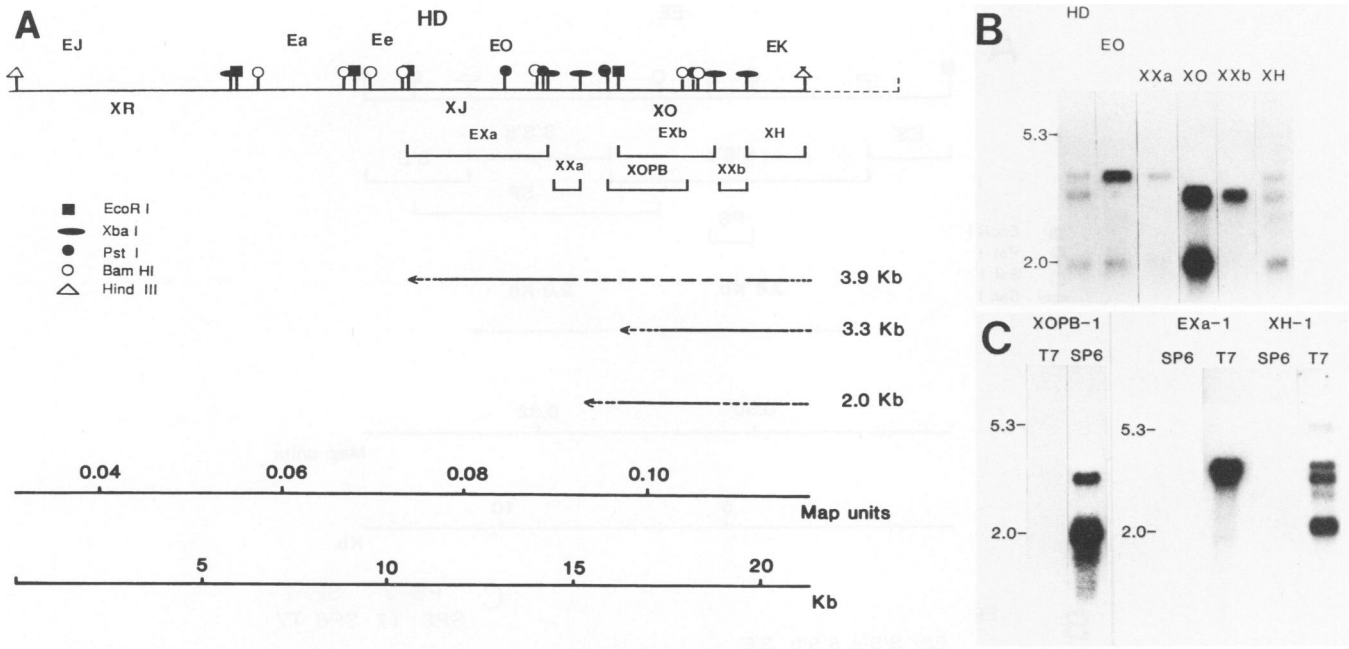

FIG. 3. Analysis of GPCMV IE RNAs encoded by region I. (A) Detailed restriction endonuclease map of region I. The *Hin*dIII, *Xba*I, *Eco*RI, *Bam*HI, and *Pst*I sites for region I were determined and are indicated on the map. The names of the *Hin*dIII and *Eco*RI fragments are listed above the line on the map, the restriction endonuclease cleavage sites are listed on the line, and the names of the subclones used for mapping transcription are indicated below the line. (B) Mapping of region I IE RNAs. The poly(A)$^+$ fraction of cytoplasmic IE RNA extracted from GPCMV-infected cells was fractionated by electrophoresis in agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled cloned GPCMV DNA fragments. Cellular 28S (5.3 kb) and 18S (2.0 kb) rRNAs were used as molecular markers. (C) Northern blot hybridization of ^{32}P-labeled riboprobes to determine direction of transcription of GPCMV IE RNAs from region I. ^{32}P-labeled riboprobes were transcribed from GPCMV DNA fragments inserted into pGEM-1 or pGEM-2 vectors. The designation SP6 or T7 indicates which transcriptase was used to prepare the riboprobe. The designation -1 or -2 following the name of the clone (e.g., EXa-1) indicates whether the clone containing the specific GPCMV DNA fragments as an insert is a pGEM-1 or pGEM-2 vector. Whether a particular GPCMV fragment is inserted in the same or opposite direction from its orientation in the genome varies depending on the restriction endonuclease sites.

GPCMV DNA fragments dotted on nitrocellulose filters. To test the validity of the procedure, we did the in vivo labeling using cells infected in the presence of cycloheximide (data not shown). When cytoplasmic RNA was extracted from the $^{32}P_i$-labeled infected cells and hybridized to cloned GPCMV DNA fragments immobilized on a nitrocellulose filter, the results were similar to those observed when radioactively labeled cDNA was used in Southern blot analysis or nick-translated DNA was used as a probe in Northern blot analysis.

We then examined GPCMV IE transcription at early times during a natural productive infection. When ^{32}P-labeled RNA was extracted from guinea pig cells infected with GPCMV for short intervals (1 or 2 h) in the absence of cycloheximide and hybridized to GPCMV DNA from the large *Hin*dIII and *Eco*RI DNA fragments and also DNA from the recombinant subclones, hybridization of RNA isolated from cells infected for 1 h to GPCMV DNA fragments containing the DNA sequences for the region II 2.0-kb transcript was significantly stronger than hybridization to the other fragments (data not shown). When RNA isolated from cells infected for 2 h was used, the DNA fragments encoding the 2.0-kb species continued to hybridize intensely and strong hybridization dots were also observed for sequences encoding the region I 3.9-, 3.3-, and 2.0-kb transcripts and the region III 2.9- and 2.7-kb transcripts. Sequences encoding the region II 3.8-kb transcript and the region III 1.9-kb transcript demonstrated hybridization, but the intensity was weaker. Hybridization was also observed to the *Hin*dIII E, *Hin*dIII I, *Eco*RI N, and *Eco*RI I DNA fragments.

Temporal expression of GPCMV IE RNAs in cells infected in the absence of cycloheximide. To obtain a more complete time course for expression of GPCMV transcripts during a natural infection, we extended the previous study beyond 2 h of infection and did dot-blot hybridization to unlabeled RNA instead of in vivo-labeled RNA (Fig. 6). RNA was extracted from uninfected cells, cells infected in the presence of cycloheximide under IE conditions, and cells infected at various times through 37 h p.i. in the absence of drug. The RNAs were blotted to nitrocellulose filters and hybridized to various ^{32}P-labeled GPCMV DNA fragments. The labeled probes included (i) fragments that identify transcription from regions I, II, and III, (ii) *Hin*dIII-E, (iii) *Hin*dIII-I, (iv) *Eco*RI-N, (v) *Eco*RI-I, and (vi) a probe (*Hin*dIII-N) that does not contain sequences that encode IE transcripts but does contain sequences that encode an early transcript(s). The results of these studies were in agreement with the in vivo labeling data and showed that the region II *Eco*RI E 2.0-kb transcript is the most abundant transcript identified at 1 h after GPCMV infection in the absence of cycloheximide. It is also apparent from these studies that expression of the region II *Eco*RI E 2.0-kb transcript is transient and diminishes markedly by 13 h p.i., expression of some of the IE transcripts, such as the region II transcripts, is maximal at earlier times than others (Table 1), and expression of an early transcript(s) encoded by sequences

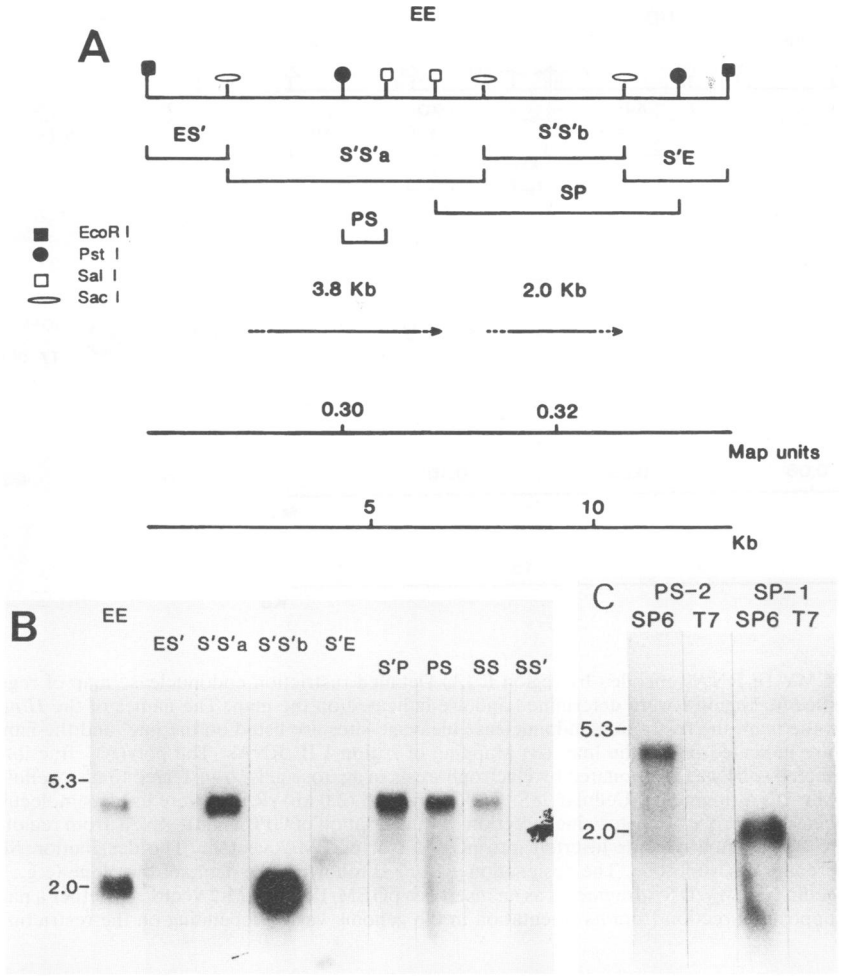

FIG. 4. Analysis of GPCMV IE RNAs encoded by region II. (A) Detailed restriction endonuclease map of region II. The *Eco*RI, *Sac*I, *Sal*I, and *Pst*I sites for region II were determined and are indicated on the map. The *Sac*I restriction endonuclease sites are indicated by S′ to distinguish them from the *Sal*I (S) sites. The restriction endonuclease cleavage sites are listed on the line, and the names of the subclones used for mapping transcription are indicated below the line. (B) Mapping of region II IE RNAs. The poly(A)$^+$ fraction of cytoplasmic IE RNA extracted from GPCMV-infected cells was fractionated by electrophoresis in agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled cloned GPCMV DNA fragments spanning region II. The DNA fragments S′P, SS, and SS′ were not cloned and were therefore not included in the map in panel A of this figure. The location of these fragments within region II can be determined from the map in panel A by using the symbols for the specific restriction sites. (C) Northern blot hybridization of ^{32}P-labeled riboprobes to determine direction of transcription of GPCMV IE RNAs from region II was done as described in the legend to Fig. 3C.

from *Hin*dIII-N was not detected under IE conditions or at 1 and 2 h p.i. in the absence of drug but was detected by 3 to 5 h p.i. in the absence of drug. The intensity of hybridization was particularly strong when the S′S′b and PB probes were used for hybridization. The S′S′b probe hybridizes to only one transcript, the region II 2.0-kb transcript, while the PB probe hybridizes to two transcripts, the region III 3.6- and 2.7-kb transcripts.

Specificity of DNA sequences that encode IE transcripts from regions I, II, and III. Small GPCMV DNA fragments that contain sequences which code for GPCMV IE RNA were used in Northern analyses to determine whether these fragments are IE specific or also encode sequences for GPCMV early or late transcripts (Fig. 7). None of the IE transcripts continued to be expressed strongly at early times, but some were expressed weakly. Two probes hybridized strongly and many of the probes hybridized weakly to late RNAs of different size classes, indicating that the sequences contained in these probes also encode portions of specific late transcripts. The probe which hybridizes to the 2.0-kb transcript from region II was particularly IE specific and did not hybridize to early or late RNAs.

Northern blot analysis to identify IE transcripts encoded by *Hin*dIII E, *Hin*dIII I, *Eco*RI N, and *Eco*RI I DNA fragments. It was apparent from the in vivo labeling experiments and time course studies that the *Hin*dIII E, *Hin*dIII I, *Eco*RI N, and *Eco*RI I DNA fragments in addition to regions I, II, and III DNA fragments hybridized to IE RNA. Northern blot analyses confirmed these findings and made it possible to determine the number and approximate size classes of RNAs identified by each fragment (Fig. 8). The nine transcripts from regions I, II, and III that were previously identified and characterized in detail were observed when hybridization was done with the *Hin*dIII D, *Eco*RI E, and *Hin*dIII B DNA fragments. At least eight additional transcripts were also identified including a particularly abundant transcript encoded by *Hin*dIII-I. *Hin*dIII-E codes for at least two and *Hin*dIII-I for at least three transcripts. *Eco*RI-N encodes one

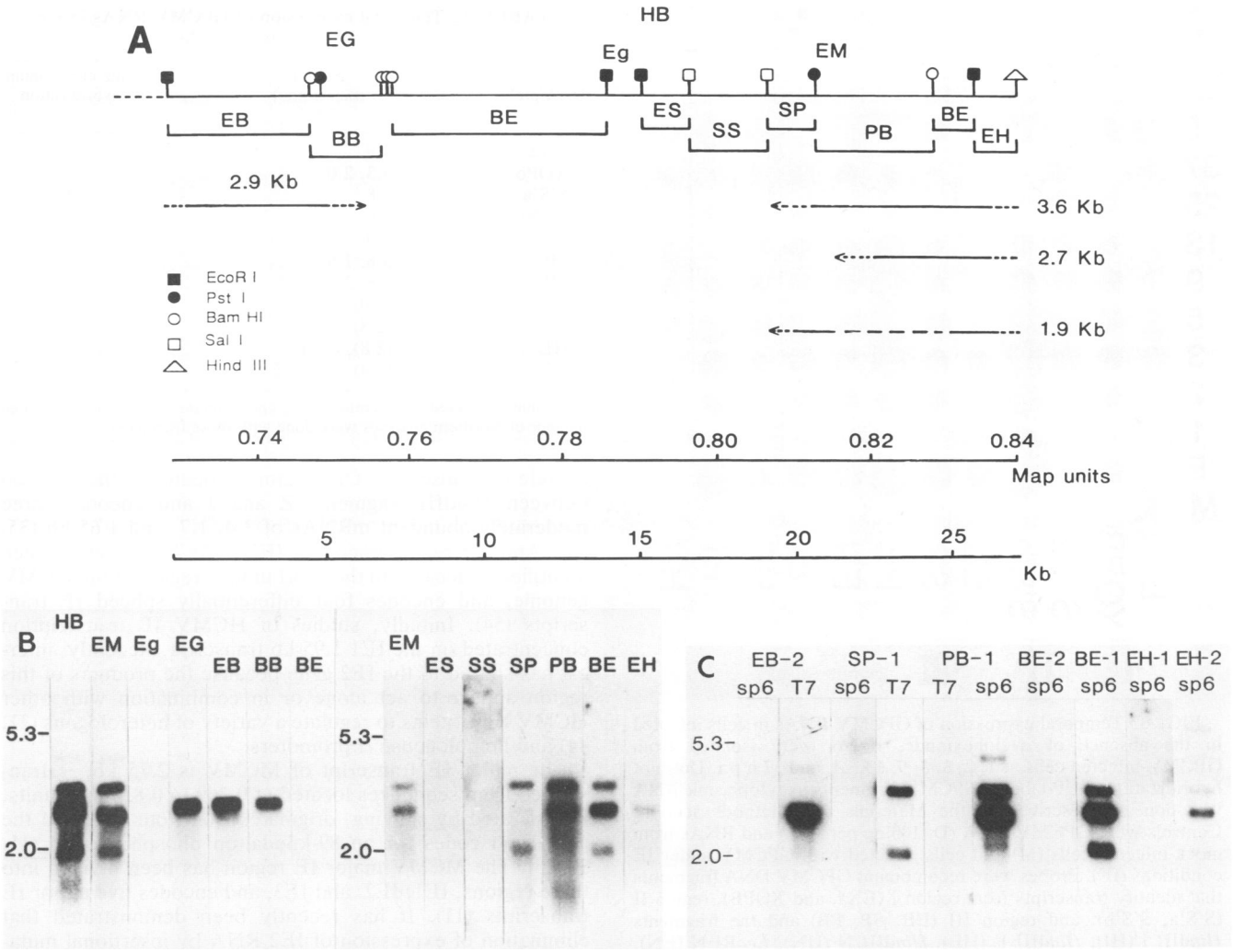

FIG. 5. Analysis of GPCMV IE RNAs encoded by region III. (A) Detailed restriction endonuclease map of region III. The *Hin*dIII, *Eco*RI, *Bam*HI, *Pst*I, and *Sal*I sites for region III were determined and are indicated on the map. The names of the *Hin*dIII and *Eco*RI fragments are listed above the line on the map, the restriction endonuclease cleavage sites are listed on the line, and the names of the subclones used for mapping transcription are indicated below the line. (B) Mapping of region III IE RNAs. The poly(A)$^+$ fraction of cytoplasmic IE RNA extracted from GPCMV-infected cells was fractionated by electrophoresis in agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled cloned GPCMV DNA fragments. (C) Northern blot hybridization of ^{32}P-labeled riboprobes to determine direction of transcription of GPCMV IE RNAs from region III was done as described in the legend to Fig. 3C.

large transcript, and *Eco*RI-I encodes at least two transcripts.

DISCUSSION

In the herpesviruses, including the CMVs, virus gene expression is divided into at least three phases: (i) the IE phase observed in the absence of protein synthesis, (ii) the early phase, which occurs before the onset of virus DNA synthesis, and (iii) the late phase, which occurs after the onset of virus DNA replication. In this study, we initiated an analysis of GPCMV gene expression, in particular, GPCMV IE gene expression, during productive infection of guinea pig cells in culture. We conclude the following from these studies. (i) GPCMV is temporally regulated, with gene expression being more limited under IE conditions than under early or late conditions. (ii) GPCMV IE RNA is transcribed from several distinct regions of the GPCMV genome, and at least 17 different RNA species can be identified. (iii) Nine IE transcripts that are transcribed from three distinct regions of the genome designated regions I, II, and III were mapped. (iv) Three of the IE transcripts from region I and three from region III were transcribed in the same direction from overlapping sequences. (v) The region II *Eco*RI E 2.0-kb transcript is the most abundant GPCMV transcript detected in the presence of cycloheximide and the most abundant transcript identified at 1 h after GPCMV infection in the absence of cycloheximide. (iv) The subclone that was used to identify the region II *Eco*RI E 2.0-kb transcript hybridizes only to GPCMV IE RNA and does not hybridize to GPCMV early or late RNA.

In our previous studies, we prepared more than 40 recombinant DNA clones containing most of the GPCMV *Hin*dIII and *Eco*RI DNA fragments (16). These clones were used to generate *Hin*dIII, *Eco*RI, and *Xba*I restriction endonuclease maps of the entire GPCMV genome. Many of the *Hin*dIII and *Eco*RI DNA fragments are large and were useful initially

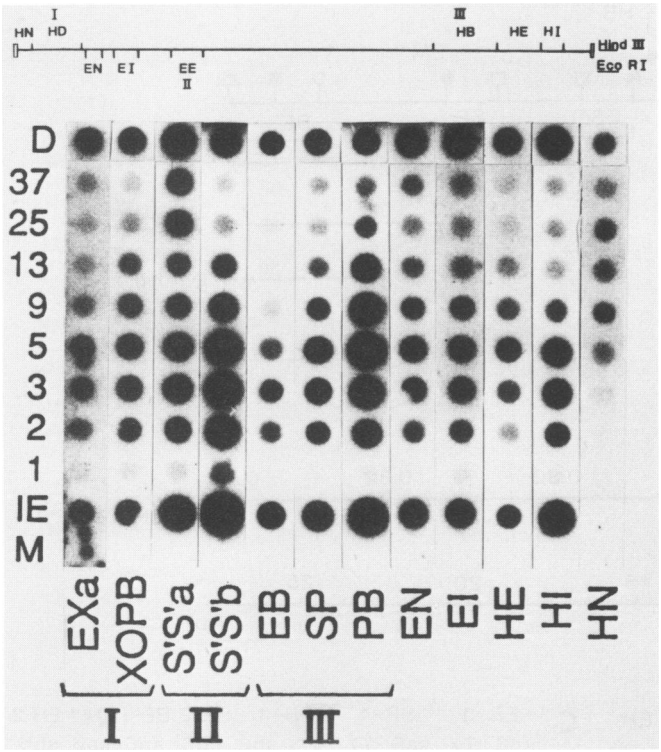

FIG. 6. Temporal expression of GPCMV RNAs in cells infected in the absence of cycloheximide. RNAs were isolated from GPCMV-infected cells at 1, 2, 3, 5, 9, 13, 25, and 37 h p.i. Dot-blot hybridization of ^{32}P-labeled GPCMV fragments to cytoplasmic RNA was done as described in the Materials and Methods section. Controls were GPCMV DNA (D, 100 ng per spot) and RNAs from mock-infected cells (M) and cells infected with GPCMV under IE conditions (IE). Probes were recombinant GPCMV DNA fragments that identify transcripts from region I (EXa, and XOPB), region II (S'S'a, S'S'b), and region III (EB, SP, PB) and the fragments *Hin*dIII I (HI), *Hin*dIII E (HE), *Hin*dIII N (HN), *Eco*RI N (EN), and *Eco*RI I (EI). *Hin*dIII-N (HN) does not contain sequences that encode IE transcripts but does contain sequences that encode early transcript(s) and was used as a control.

in the current studies for scanning the GPCMV genome for the regions that contain sequences which encode IE, early, and late transcripts. During the current study, we generated more than 20 subclones containing small GPCMV DNA fragments. We used these subclones to map specific GPCMV IE transcripts and in so doing we generated detailed restriction endonuclease maps of the *Hin*dIII B and D and the *Eco*RI E fragments. These subclones can be used in future studies to hybrid select specific transcripts for in vitro translation to determine what proteins they encode, and they can also be utilized in in situ hybridization to identify expression of specific transcripts at the cellular level.

HCMV (27, 49–51, 54) and murine CMV (MCMV) (11, 30–32) IE transcription has been characterized extensively. The major IE transcript of HCMV (IE1) is 1.95 kb, is encoded by sequences located at 0.739 to 0.755 map units of the long unique sequence (within the *Hin*dIII E fragment), and encodes a 72-kilodalton phosphoprotein. Sequences just to the left of this region are also transcribed under IE conditions (the IE2 gene). At least six overlapping spliced IE RNAs can be transcribed from the IE1 and IE2 genes. There are two additional regions of the HCMV genome in addition to the IE1 and IE2 genes which contain sequences that

TABLE 1. Temporal expression of GPCMV RNAs in the absence of cycloheximide

DNA probe	Size of transcript(s) (kb)[a]	Time of maximum hybridization (h p.i.)
EXa	3.9	5
XOPb	3.3, 2.0	3
S'S'a	3.8	3
S'S'b	2.0	3
EB	2.9	3
SP	3.6, 1.9	5
PB	3.6, 2.7	5
EN	(6.0)	5
EI	(2.8), (1.8)	5
HE	(2.8), (2.5)	5
HI	(3.4), (2.7), (2.5)	3

[a] Values indicated in parentheses are approximate because only a limited number of Northern analyses were done with these fragments.

encode IE transcripts. One region is located at the junction between *Hin*dIII fragments Z and J and encodes three moderately abundant mRNAs of 3.4, 1.7, and 1.65 kb (35, 55). Another region encoding IE RNAs has recently been identified, is located in the short unique region of the HCMV genome, and encodes four differentially spliced IE transcripts (54). Initially, studies of HCMV IE transcription concentrated on the IE1 1.95-kb transcript. Recently, interest has shifted to the IE2 gene because the products of this region appear to act alone or in combination with other HCMV IE proteins to regulate a variety of heterologous (21, 44) and homologous (7) promoters.

The major IE transcript of MCMV is 2.75 kb, is transcribed from sequences located at 0.769 to 0.815 map units, is generated by splicing, originates from four exons in the gene, and codes for an 89-kilodalton phosphoprotein (11, 30–33). The MCMV major IE region has been divided into three regions, IE1, IE2, and IE3, and encodes five minor IE transcripts (11). It has recently been demonstrated that elimination of expression of IE2 RNA by insertional mutagenesis has no effect on the ability of MCMV to replicate in cells in culture (38). Low levels of MCMV IE transcription have also been detected from both termini of the genome (39).

IE virus genes have been shown to play regulatory roles. IE genes can contain regulatory sequences that respond to transactivating factors that initiate virus replication, and IE gene products can also act to positively or negatively regulate the expression of other virus genes so that the replication cycle can proceed. These kinds of regulatory patterns have been observed for several herpes simplex virus IE genes (6, 12, 13, 17, 36, 40, 43, 45, 53). The HCMV IE2 gene alone or in combination with the IE1 gene can transactivate expression from heterologous and homologous promoters (7, 10, 21, 44, 52), suggesting that HCMV IE genes like herpes simplex virus genes are important regulatory genes. It has also been demonstrated that both HCMV and MCMV IE genes contain strong enhancer sequences, suggesting that these sequences play critical roles in responding to regulatory factors. Molecular studies of GPCMV to date have been limited, and to our knowledge, there have been no published reports concerning expression of GPCMV RNAs. To understand GPCMV replication, it will be necessary to analyze expression of many GPCMV genes including genes that are expressed at IE, early, and late times. We initiated our analysis of GPCMV gene expression by beginning to characterize some of the GPMCV IE transcripts because previ-

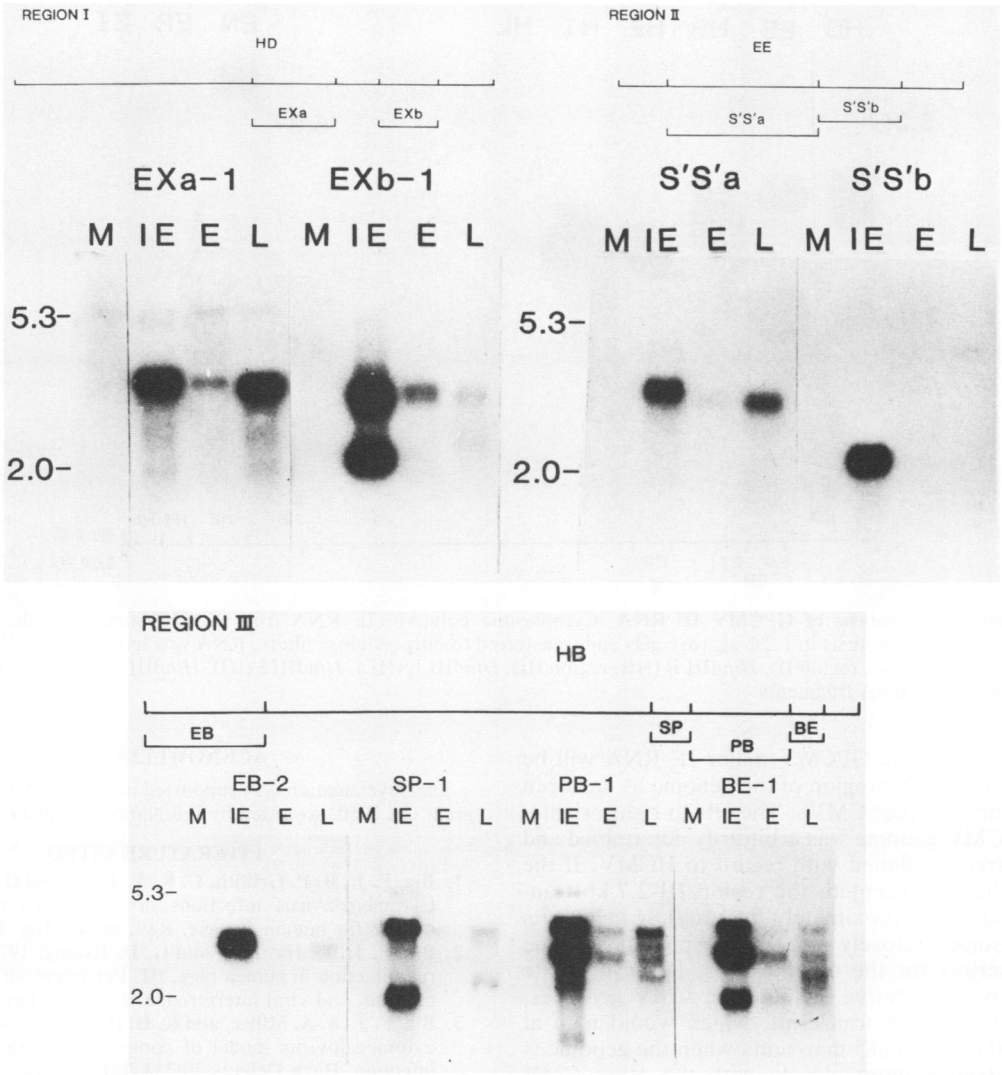

FIG. 7. Northern blot hybridization of GPCMV DNA subfragments to GPCMV IE, early, and late RNA. RNAs from uninfected cells (M), IE, early (E), and late (L) GPCMV RNAs were fractionated by size in agarose gels, transferred to nitrocellulose filters, and hybridized to two probes that identify region I IE RNA (EXa and EXb), two probes that identify region II IE RNA (S'S'a and S'S'b), and four probes that identify region III IE RNA (EB, SP, PB, and BE).

ous studies have shown that IE virus genes may play critical regulatory roles.

It has previously been reported that the HCMV IE1 gene is only expressed immediately after infection (48). In contrast, the MCMV IE1 gene is transcribed at IE times and at late times but not at early times; similarly, the corresponding MCMV IE 89-kilodalton protein is reexpressed at late times. It has recently been shown that the HCMV region 2 which codes for a series of differentially spliced IE transcripts also codes for a 1.5-kb transcript that is transcribed from a late promoter only at late times p.i. (47). The 1.5-kb transcript encodes a 40-kilodalton protein that is also expressed only at late times p.i. In this study, we showed that the GPCMV region II 2.0-kb transcript which is transcribed abundantly at IE times is not transcribed at early times or at late times, suggesting that it most closely resembles HCMV IE1 in this property. We also observed that abundant late transcripts were identified using the DNA probes that hybridize to the GPCMV region I 3.9-kb transcript and the region II 3.8-kb transcript. These late transcripts differ slightly in size from the IE transcripts identified by the same probes. Detailed mapping of these late RNAs will have to be done before it can be determined whether they and the IE RNAs are transcribed from overlapping sequences and whether they share promoters.

Comparison of what we know to date about GPCMV IE transcription with what is known about HCMV and MCMV IE transcription indicates some similarities. One strong similarity between GPCMV, HCMV, and MCMV IE transcription is the existence of overlapping differentially spliced IE transcripts. A second is that all three viruses express a reasonably large number of IE RNAs. To date we have not identified the most significant GPCMV major IE transcripts. Two likely candidates with regard to abundance are the region II 2.0-kb transcript and the region III 2.7-kb transcript. It has been shown that the HCMV and MCMV major IE transcripts are encoded by sequences located at 0.739 to 0.755 map units and 0.769 to 0.815 map units, respectively. Similarly, the IE94 gene of simian CMV (strain Colburn) maps at coordinates 0.71 to 0.73 (28). There is no a priori

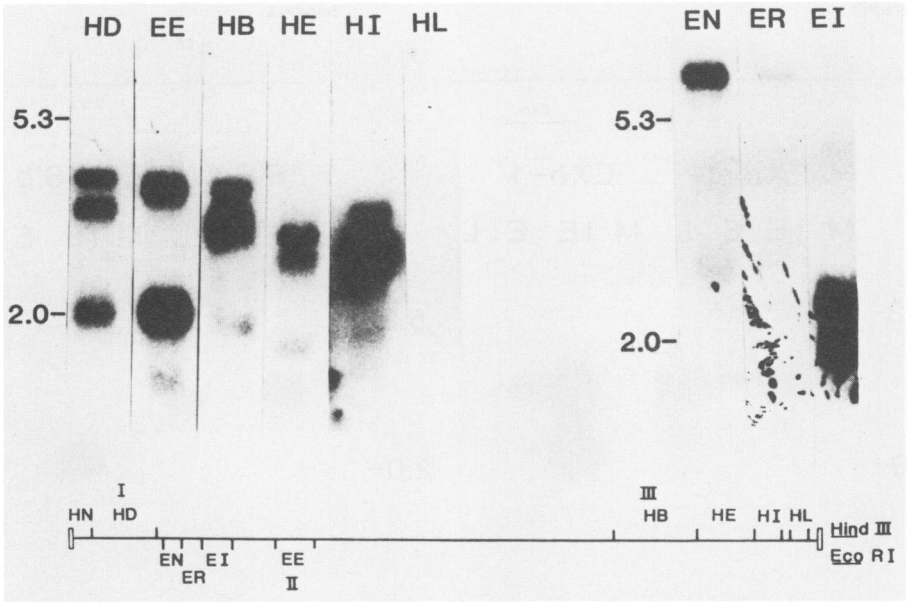

FIG. 8. Northern blot analysis of GPCMV IE RNA. Cytoplasmic poly(A)$^+$ IE RNA from GPCMV-infected cells was fractionated according to size by electrophoresis in 1.2% agarose gels and transferred to nitrocellulose filters. RNA was hybridized to ^{32}P-labeled *Hin*dIII D (HD, region I), *Eco*RI E (EE, region II), *Hin*dIII B (HB, region III), *Hin*dIII E (HE), *Hin*dIII I (HI), *Hin*dIII L (HL), *Eco*RI N (EN), *Eco*RI R (ER), and *Eco*RI I (EI) DNA fragments.

reason to believe that the GPCMV major IE RNA will be transcribed from the same region of the genome as has been demonstrated for the other CMVs. The left-to-right orientation for the GPCMV genome was arbitrarily determined and may not be correctly aligned with regard to HCMV. If the current orientation is accepted, the region III 2.7-kb transcript which maps at approximately 0.81 to 0.84 map units would align reasonably closely with the map positions for the major IE transcripts for the other CMVs. If the opposite orientation is correct relative to the other CMV genomes, then the region II 2.0-kb transcript, which would map at approximately 0.675 to 0.685 map units when the genome is inverted, would align more closely with the other CMV major IE transcripts.

We showed that there are at least 17 GPCMV IE transcripts. We observed this large number of transcripts under IE conditions as defined by cycloheximide block and also during a natural infection in the absence of drugs. In the time course analysis, transcription from all the IE regions of the genome was easily apparent by 2 h p.i. (1 h postabsorption). In addition, a rise and fall in RNA levels was observed, demonstrating the transient nature of expression of these transcripts. At this time it is not possible to identify which of the GPCMV transcripts will be the most interesting functionally. The most abundant transcript may or may not be the most significant.

We provided the foundation for understanding GPMCV IE transcription and generated a series of probes that selectively hybridize to one or at most two of the transcripts. We have not identified start sites for transcription and the exact 3′ ends for each transcript. We also have not identified the exact numbers and locations of exons and introns. The reagents we generated and the information we obtained will enable us to study IE transcription in more detail and to determine whether GPCMV IE gene products function to regulate GPCMV gene expression. These studies indicate that GPCMV IE expression during productive infection in cells in culture is complex.

ACKNOWLEDGMENT

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