

The human cytomegalovirus glycoprotein B gene (ORF UL55) is expressed early in the infectious cycle

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Northern hybridizations were carried out using mRNA preparations of human cytomegalovirus (HCMV)-infected cultures and gene-specific antisense RNA probes for transcriptional analysis of the gene cluster composed of genes for DNA polymerase, glycoprotein B (gB), herpes simplex virus-infected cell protein 18.5 homologue p130 and a major DNA-binding protein corresponding to open reading frames (ORFs) UL54–UL57, respectively. Monocistronic transcripts of 5 kb and 3.7 kb were found for ORFs UL54 and UL55, respectively, and five additional high molecular mass overlapping transcripts of 14 kb, 10 kb, 10 kb, 8 kb and 6 kb were found. Mapping of 5' ends showed that transcription was initiated at the expected distance downstream of predicted TATA elements; in the case

of a UL56-specific transcript two potential initiation sites were identified. Transcription was found to terminate at the expected distance downstream of either of two prominent polyadenylation consensus motifs in the region of UL54. All transcripts were identified early in the infectious cycle, except for the UL55 (gB)-specific transcript of 3.7 kb which was not synthesized until late post-infection. However, specific immunoreactions demonstrated the presence of a gB-specific polypeptide early after infection in the absence of viral DNA synthesis. It is suggested that a bicistronic transcript of 8 kb encoded by ORFs UL55 and UL54 is involved in biosynthesis of early HCMV gB.

Introduction

All known herpesviruses studied to date have a number of apparently essential genes which are highly conserved throughout the virus family (Kouzarides *et al.*, 1987*b*; Albrecht & Fleckenstein, 1990). Prominent with regard to its size is a block of contiguous genes (gB/POL block) consisting of open reading frames (ORFs) encoding viral DNA polymerase, glycoprotein B, infected-cell protein (ICP) 18.5 homologues of herpes simplex virus and a DNA-binding protein. The flanking regions of the gB/POL block, on the other hand, do not exhibit any obvious nucleotide sequence homologies and do not seem to code for homologous gene products. The sequential order of the four ORFs in the gB/POL gene block mentioned above has been recognized in β - and γ -herpesviruses, but differs in α -herpesvirus genomes where the DNA polymerase gene is localized adjacent to that of the DNA-binding protein and oriented in the opposite direction (Albrecht & Fleckenstein, 1990). It is noteworthy that the ORFs of this conserved gene

cluster code for products that seem to exhibit very diverse functions, i.e. two nonstructural proteins (the major single-stranded DNA-binding protein and the DNA polymerase) on the one hand, and on the other hand, two structural proteins, the ICP 18.5 homologue, which may also be a DNA-binding protein involved in DNA packaging (Mettenleiter *et al.*, 1993; Tengelsen *et al.*, 1993; E. Bogner, M. Landidini, K. Radsak & M. Stinski, unpublished results), and the gB homologue, which is a component of the viral envelope in most herpesviruses, Epstein–Barr virus (EBV) being one known exception (Gong & Kieff, 1990). The homologous products of this gene cluster appear to provide essential functions during the early phase of the infectious cycle, and in the maturation of infectious herpesvirus progeny (e.g. Herrold *et al.*, 1996; Cai *et al.*, 1988; Schleiss, 1994, 1995; Mettenleiter *et al.*, 1993; Tengelsen *et al.*, 1993). Transcription of single genes of this cluster has been studied for several herpesvirus species, and has in general revealed a complex pattern of presumably overlapping messengers (Anders & Gibson, 1988; Spaete *et al.*, 1988; Schleiss, 1995).

In the case of the β -herpesvirus human cytomegalovirus (HCMV), previous analysis of the respective gene block by

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Spaete *et al.* (1988) suggested that for all ORFs, mRNAs are transcribed unidirectionally from the complementary strand (Chee *et al.*, 1990). In addition to monocistronic transcripts for the DNA polymerase gene (UL54) and that of the gB homologue (UL55), high molecular mass most likely polycistronic transcripts were described which were not precisely assigned to specific ORFs. The present study extends these findings, providing evidence for the existence of at least seven HCMV gB/POL block-specific transcripts. Five transcription initiation sites, two of which were located upstream of ORF UL56, and two termination sites in the region of ORF UL54 were identified. An intriguing observation was the detection of an 'early' bicistronic messenger of 8 kb specific for ORFs UL55/UL54 and of a gB (gpUL55)-specific polypeptide in extracts from infected cell cultures kept in the presence of inhibitors which specifically suppress viral DNA synthesis.

Methods

■ **Cells and virus.** Monolayers of human foreskin fibroblasts (HFF) and human astrocytoma cells (U373) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS), vitamins, nonessential amino acids, glutamine, penicillin at 0.5 U/ml and gentamycin at 60 µg/ml. For propagation of HCMV strains AD169 and Towne, confluent monolayers of HFF (1.5×10^7 cells) were infected at an m.o.i. of approximately 0.1 and serum concentration was lowered to 2%; for experimental infection, an m.o.i. of about 3 was used. Virus titrations were performed by the end-point dilution method using indirect immunofluorescence for the detection of immediate early HCMV antigen with a commercial MAb (Dupont; Radsak *et al.*, 1990).

■ **Preparation of antisense RNA probes.** As a first step the following restriction enzyme fragments of cosmid 1029 (Fleckenstein *et al.*, 1982; Kollert-Jöns *et al.*, 1991) were subcloned into pUC18 in order to obtain specific amplification constructs for ORFs UL54 through 57:

pUC18-UL54 was obtained by subcloning the 5.2 kb *PvuII*-*SacI* fragment of cosmid 1029 into the vector after digestion with *SmaI/SacI*; pUC18-UL55 was constructed by subcloning the 3.1 kb *XmaIII* fragment of cosmid 1029 into the *XmaIII* site of the vector (Reis *et al.*, 1993); the 3.4 kb *HpaI*-*SalI* fragment of cosmid 1029 was inserted into the *SmaI/SalI* restriction sites of the vector to obtain pUC18-UL56 (Bogner *et al.*, 1993); and in the case of pUC18-UL57 the 3.9 kb *SmaI*-*XhoI* fragment of cosmid 1029 was subcloned into the vector after digestion with *SmaI/SalI*. Correct subcloning of the fragments was examined by sequencing the sites of insertion (Sanger *et al.*, 1977). Five defined restriction fragments representative of UL54, the intervening sequence between UL54/55, UL55, UL56 and UL57 were excised from the viral inserts of the four pUC18 constructs (Table 1) and transferred into the multiple cloning sites of vectors pGEM-3Zf(+) or pGEM-11Zf(+). Following digestion of transcription constructs as described in Table 1, 'run off' transcription under the control of the T7 or SP6 promoter was performed in the presence of digoxigenin (DIG)-11-UTP according to the manufacturer's instructions (Boehringer Mannheim). For preparation of the glyceraldehyde phosphate dehydrogenase (GAPDH) probe, which was used to estimate mRNA quantities in the various samples via reaction of the constitutively expressed GAPDH transcript, an appropriate pCR II-construct (Invitrogen) was digested with *Bam*HI prior to antisense transcription under the control of the T7 promoter. The UL75 (glycoprotein H gene)-specific probe was prepared by isolation of a 410 bp fragment after digestion of a pRC/CMV-UL75 construct with *Bgl*II and *Bam*HI and insertion of the fragment in antisense orientation into pcDNA3; following linearization of the pcDNA3-asUL75 construct with *Bam*HI the probe was obtained after transcription under the control of the T7 promoter. The DIG-labelled antisense RNA probes produced under these conditions consisted of molecules of uniform length and were used for Northern blotting with infected-cell mRNA (see below).

■ **Preparation of infected-cell mRNA and Northern blotting.** For Northern blotting, total cell RNA was extracted following the method of Chomczynski & Sacchi (1987) as modified by Kollert-Jöns *et al.* (1991). Briefly, 1.5×10^7 mock- or HCMV-infected HFF or U373 cells (m.o.i. approximately 3) were lysed at various times post-infection (p.i.;

Table 1. Preparation of gene-specific RNA probes

Appropriate pUC18 constructs (see Methods) were digested with the indicated restriction endonucleases (columns '3' end' and '5' end') and gene-specific fragments were isolated (column 'Size of gene-specific fragments') prior to insertion in the correct orientation into the multiple cloning sites of pGEM-3Zf(+) and pGEM-11Zf(+) (column 'Transcription vector'), respectively, using restriction enzymes listed in columns 'T7' or 'SP6', respectively. For transcription of antisense RNA probes under the control of the T7 promoter, pGEM constructs were digested with *Bam*HI in the case of pGEM-UL54, -UL54/55, -UL55, -UL57, or with *Hind*III in the case of pGEM-UL56; for transcription of sense RNA probes under the control of the SP6 promoter, digestion was performed with *Bam*HI for pGEM-UL54, -UL54/55, *Eco*R1 for pGEM-UL55, -UL57, and with *Bgl*II for pGEM-UL56.

Transcription vector	RNA probe		Size (bp) of gene-specific fragments excised from the respective pUC18 constructs	5' end		
	T7	3' end specific for:		SP6	SP6	
pGEM-11	<i>Apa</i> I	<i>Apa</i> I	UL54	1175	<i>Pst</i> I	<i>Nsi</i> I
pGEM-11	<i>Not</i> I	<i>Not</i> I	UL54/55	470	<i>Nsi</i> I	<i>Nsi</i> I
pGEM-3	<i>Eco</i> RI	<i>Eco</i> RI	UL55	710	<i>Cl</i> aI	<i>Acc</i> I
pGEM-11	<i>Sac</i> I	<i>Sac</i> I	UL56	880	<i>Xho</i> I	<i>Sal</i> I
pGEM-3	<i>Sac</i> I	<i>Sac</i> I	UL57	1470	<i>Sal</i> I	<i>Sal</i> I

Table 2. Genomic positions and nucleotide sequence of primers used for mapping of 5' and 3' ends

(a) Gene specific primers used for 5' end determination

ORF	Reverse transcriptase reaction		Polymerase chain reaction		Sequencing reaction	
	Genomic bp position	Distance from +1 (bp)	Genomic bp position	Distance from +1 (bp)	Genomic bp position	Distance from +1 (bp)
UL54	80635–80664	345	80790–80814	195	80825–80849	160
UL55	83140–83165	688	83464–83488	365	83464–83488	365
UL56-1	85330–85355	740	85915–85939	155	85915–85939	155
UL56-2	85330–85355	697	85915–85939	113	85915–85939	113
UL57	89484–89512	790	90040–90064	238	90040–90064	238

(b) G- and C-tail directed primers used for 5' end determination

Tail	Nucleotide sequence
C	CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIG*
G	CUACUACUACUAGGCCACGCGTCGACTAGTACGCCCCCCCCCCCCCCCC

* Purchased from GIBCO Life Technologies.

(c) Gene specific primers used for 3' end determination

ORF	Nucleotide sequence	Genomic bp position	Distance from 3' end (bp)
UL54	CGTGAGCATCTGCGTGATGCAGTCGCAG	76460–76433	185
UL55	CGTGCCGCGAGGTGTCATGTTTCGACGGT	80501–80473	78

see Results) in 4 ml Sol D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) *N*-lauroyl sodium sarcosinate, 0.1 M 2-mercaptoethanol]. After the addition of 0.5 ml 2 M sodium acetate (pH 4.0), RNA was extracted by vigorous shaking of the mixture with 5 ml equilibrated phenol and 1 ml chloroform–isoamyl alcohol (24:1) for 10 s. After incubation on ice for 15 min, the emulsion was centrifuged for 15 min at 10000 r.p.m. and 4 °C (SW41 rotor, Beckman L8-M ultracentrifuge). The aqueous phase was collected and the RNA was precipitated overnight with 5 ml 2-propanol. To protect the RNA from degradation by contaminating RNases, RNase inhibitor (Boehringer Mannheim) was added to the aqueous phase at 5 U/ml according to the instructions of the supplier. Poly(A)⁺ mRNA was purified from total cell RNA using oligo(dT)-affinity resin (Oligotex mRNA kit, Qiagen) following the manufacturer's instructions. For quantification of mRNA samples absorbance at 260 nm was determined.

Formaldehyde–agarose gel electrophoresis of mRNAs and Northern blotting was carried out according to methods described by Sambrook *et al.* (1989) using Hybond-N+ membrane (Amersham) for mRNA transfer. For hybridization of the blotted and cross-linked mRNAs and the DIG-labelled antisense RNA probes the following protocol was used: the blot membrane was incubated for at least 1 h at 68 °C in prehybridization buffer [5 × SSC (0.15 M NaCl, 0.015 sodium citrate, pH 7.0), 50%

formamide, 0.02% SDS, 0.1% lauroyl sarcosinate, 2% blocking reagent (Boehringer Mannheim) in 100 mM maleic acid, 150 mM NaCl, pH 7.5]. The prehybridization mixture was subsequently replaced by fresh prehybridization buffer supplemented with the heat-denatured DIG-labelled antisense RNA probe (100 °C for 10 min) for hybridization overnight at 68 °C. After washing the blot membranes (twice for 10 min in 2 × SSC containing 0.1% SDS at room temperature and twice for 15 min in 0.1 × SSC containing 0.1% SDS at 68 °C), detection of hybridized DIG-labelled probes by means of X-ray films was achieved by incubation with anti-DIG F(ab)₂ fragments conjugated to alkaline phosphatase using a DIG luminescent detection kit following precisely the manufacturer's instructions (Boehringer Mannheim).

■ **Mapping of 5' and 3' ends of gB/POL block-specific mRNA.** cDNAs were prepared by extension of gene-specific oligonucleotides hybridized to isolated mRNAs using a commercial RACE (rapid amplification of cDNA ends) kit (GIBCO Life Technologies). The sequences of the oligonucleotides used for the extension reactions are listed in Table 2. 5'-end cDNAs were subsequently extended with terminal nucleotide transferase by cytosine- or guanosine-tailed anchors prior to amplification using an anchor-directed primer complementary to the homopolymeric tail; amplification of 3'-end cDNAs was carried out with oligo(dT) as the second primer. Amplified cDNAs were analysed by

sequencing (Sanger *et al.*, 1977) using an automated sequence analyser (ABI PRISM 377 DNA Sequencer, Perkin Elmer). For precise localization of 5' and 3' ends, respectively, the pretail-sequences obtained for the cDNAs were aligned with that of the viral genomic sequence as determined with the first primers mentioned above (Table 2).

■ **Immunofluorescence and immunoprecipitation.** For immunofluorescence, cells were grown on round cover-slips in Petri dishes. At the desired times p.i. cover-slip cultures were fixed in 4% paraformaldehyde in PBS at room temperature for 20 min, after washing in PBS quenched with 25 mM NH₄Cl for 5 min, and permeabilized by treatment with 0.5% Triton X-100. Staining for HCMV major capsid protein (MCP) was carried out after extensive rinsing in PBS with murine MAb 28-4 (provided by B. Plachter, Erlangen, Germany) for 60 min at room temperature prior to further incubation at room temperature for 60 min with fluorescein-labelled goat anti-mouse F(ab)₂ fragments (Dianova). For double staining a mixture of MAbs against the gene product of HCMV UL112/113, which exhibits nuclear localization (murine MAb provided by K. Hirai, Tokyo, Japan; Iwayama *et al.*, 1994), and human HCMV gB-specific MAb ITC88 (provided by M. Ohlin, Lund, Sweden; Ohlin *et al.*, 1993) or gH-specific polyclonal antibody (PAb; Bogner *et al.*, 1992) was used in the first incubation; fluorescein-labelled anti-human and Texas red-labelled anti-mouse F(ab)₂ fragments from goat (Dianova) were used in the second incubation. In the case of MAbs the first incubation was carried out with undiluted culture medium containing 10% FCS. Results were obtained using a Zeiss microscope with photographic equipment (Axiophot).

For immunoprecipitation, total cell extracts were prepared from [³⁵S]-methionine-labelled cultures (see text) by solubilization in extraction buffer (20 mM Tris-HCl, pH 9, 0.3 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10% glycerol, 1 mM PMSF; 500 ml per 5 × 10⁶ cells) and ultrasonic treatment (3 × 10 s at maximum setting in the sonification bath of a Branson Sonifier); insoluble material was sedimented for 15 min at 1500 × g. Comparable total amounts of protein of the extract samples and a mixture of gB-specific MAbs 27-156 (Spaete *et al.*, 1988) and ITC88 (Ohlin *et al.*, 1993) were used for precipitation as described previously (Bogner *et al.*, 1992), prior to separation of precipitates by SDS-PAGE (Laemmli, 1970) and fluorography.

Results

Identification of specific transcripts of ORFs UL54–57 (HCMVgB/POL block)

Because early as well as late viral gene products are encoded by ORFs of the HCMV gB/POL block, initial analyses were carried out with HCMV strain AD169-infected U373 cells at 96 h p.i. when transcription of all ORFs was most likely completed. At this time mRNA was isolated and electrophoretically separated; Northern blotting was performed as described in Methods. The approximate locations of the five gene-specific antisense RNA probes used within the respective ORFs are shown in Fig. 1(a). The probe specific for UL54 recognized four RNA species (5 kb, 8 kb, about 10 kb and 14 kb); that for UL54/55 recognized six RNA species (3.7 kb, 5 kb, 6 kb, 8 kb, approximately 10 kb and 14 kb); that for UL55 recognized five RNA species (3.7 kb, 6 kb, 8 kb, about 10 kb and 14 kb); that for UL56 reacted with three RNA species (6 kb, approximately 10 kb and 14 kb); and two signals for RNA species of about 10 kb and 14 kb were obtained with

the probe specific for UL57 (Fig. 1b). All probes except that derived from UL57 gave an additional weak signal of about 5.5 kb. Strong signals at about 5 kb for RNA from mock-infected cells were also observed only with the probes specific for UL56 and UL57, most likely representing artifactual binding to residual 28S RNA. Northern blot analyses carried out under identical conditions with mRNAs isolated from HFF cells infected with HCMV strain AD169 or strain Towne-infected U373 cells late p.i. yielded essentially identical results (data not shown) disregarding differences in signal intensities particularly for the high molecular mass transcripts which were not further investigated at this point. As an additional control, sense RNA probes whose transcription is regulated by the SP6 promoter of the pGEM constructs were prepared (Table 1) and used for Northern blotting with mRNAs from AD169-infected HFF cells; this experimental set-up yielded no specific hybridization signals.

For an interpretation of these observations, a search (GCC program) of the gB/POL block nucleotide sequence for prominent consensus motifs known to regulate transcription initiation and termination was performed, the result of which is schematically depicted in Fig. 1(a). This analysis revealed TATA elements upstream of each of the four ORFs, and several polyadenylation signals in an appropriate context with regard to the ORFs (Fig. 1a). Regarding the patterns of transcripts obtained with the various probes it is feasible to assume that transcription may be initiated downstream of each of the four predicted promoter consensus motifs, and termination may be regulated only by two of the predicted polyadenylation consensus motifs, i.e. those downstream of UL55 and UL54, respectively. Transcriptional activity would thus produce a total of seven messengers: three pairs of 5' co-terminal mRNAs representative of ORFs UL57–54 and UL57–55, ORFs UL56–54 and UL56–55, and ORFs UL55–54 and UL55, respectively, and an additional messenger for ORF UL54. The transcripts representing UL54 and UL55 should be monocistronic, and of the five polycistronic transcripts, two should have similar sizes of about 10 kb. This likely interpretation for the prominent transcripts presented in Fig. 1(b) is schematically depicted in Fig. 1(c). As the less prominent transcript of 5.5 kb was synthesized only late p.i. (see analysis of the time-course of appearance of the mRNA species) it was not considered in this context.

Mapping of the 5' and 3' ends of gB/POL-specific transcripts

As pointed out above, interpretation of the Northern hybridization analysis was based on the arrangement of potential regulatory elements in the viral genomic sequence. To confirm that transcription cap sites upstream of each ORF and termination sites, respectively, were located within the expected context, and to re-examine the estimated sizes of the observed transcripts, mapping of the 5' and 3' ends of mRNAs

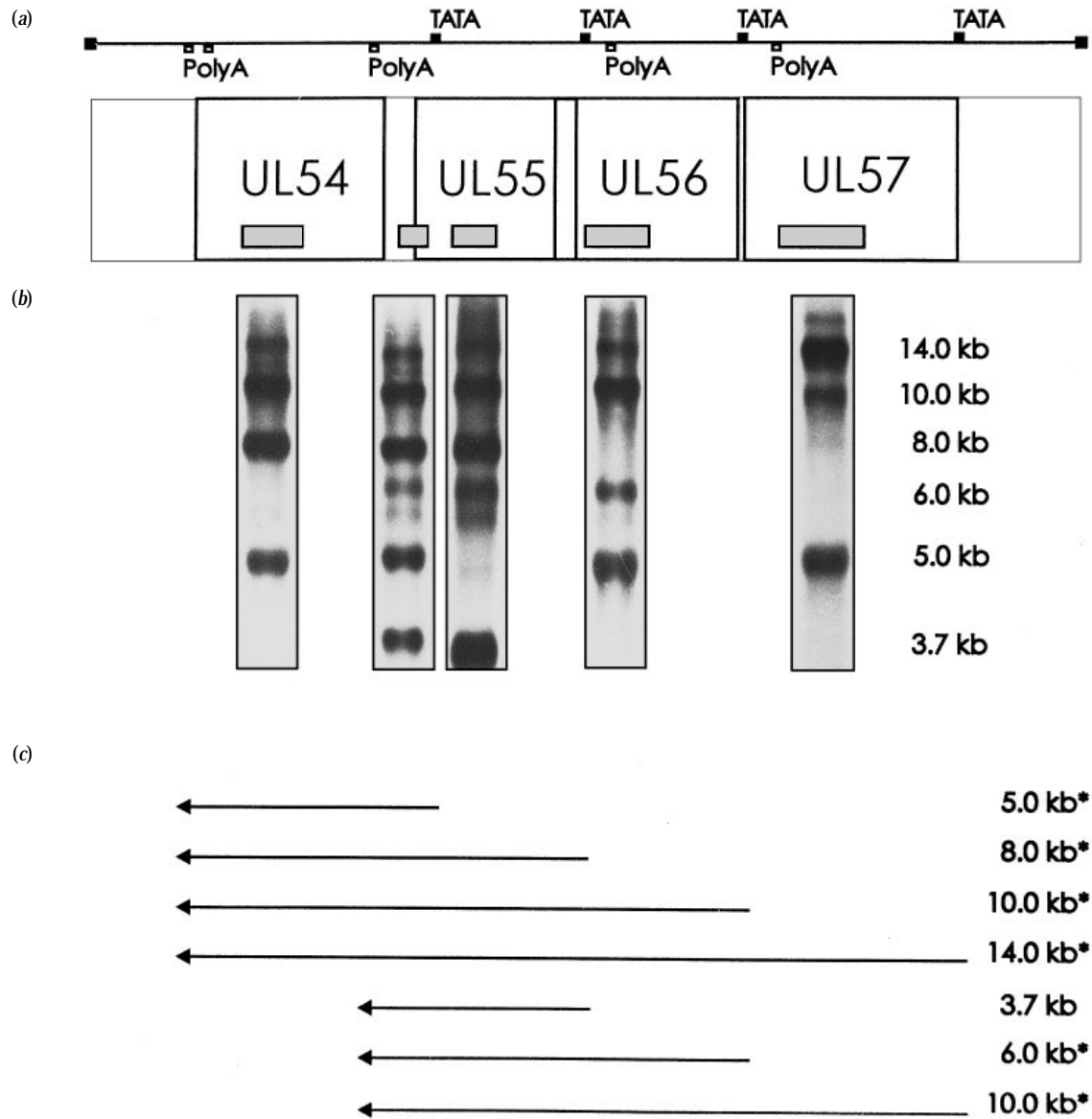


Fig. 1. Identification of mRNAs transcribed from the HCMV gB/POL (UL54–57) gene cluster. (a) Scheme of the gene cluster showing the relative sizes and locations of the RNA probes used (shaded inserts inside ORFs). Relative positions of prominent predicted TATA elements and transcription termination consensus motifs are also indicated. (b) Northern blots obtained with mRNA preparations isolated 96 h p.i. from HCMV strain AD169-infected HFF cells and gene-specific antisense RNA probes (see Table 1 in Methods). Blots are aligned underneath the respective probes indicated in (a). Sizes of the recognized transcripts are indicated on the right. UL56- and UL57-specific probes produced an artifactual signal of 5 kb most likely because of reaction with 28S rRNA. (c) Schematic interpretation of the transcription patterns presented in (b) by alignment of the various transcripts with the gene cluster in (a). The asterisk indicates sizes of mRNAs observed early p.i.

isolated at 96 h p.i. was carried out using primers for cDNA synthesis which hybridized at a distance of about 345–790 nt from the putative mRNA 5' and 3' ends, respectively (described in Methods, Table 2).

The precise transcription start sites downstream of the respective prominent TATA elements were located within an expected distance of 23–32 nt, except for ORF UL56 where a second potential start site was found another 40 nt further downstream. The genomic nucleotide sequence contains a

potential upstream TATA element at a reasonable distance of 19 nt.

Using C- as well as G-tailing of 5' end cDNAs prior to amplification, single terminating C-residues were found that did not conform to the viral genomic sequence. A likely interpretation is that these additional residues originated from transcription of the cap structure nucleotides during cDNA synthesis. Regarding termination, transcription was found to proceed to 16 nt downstream of either of the two prominent

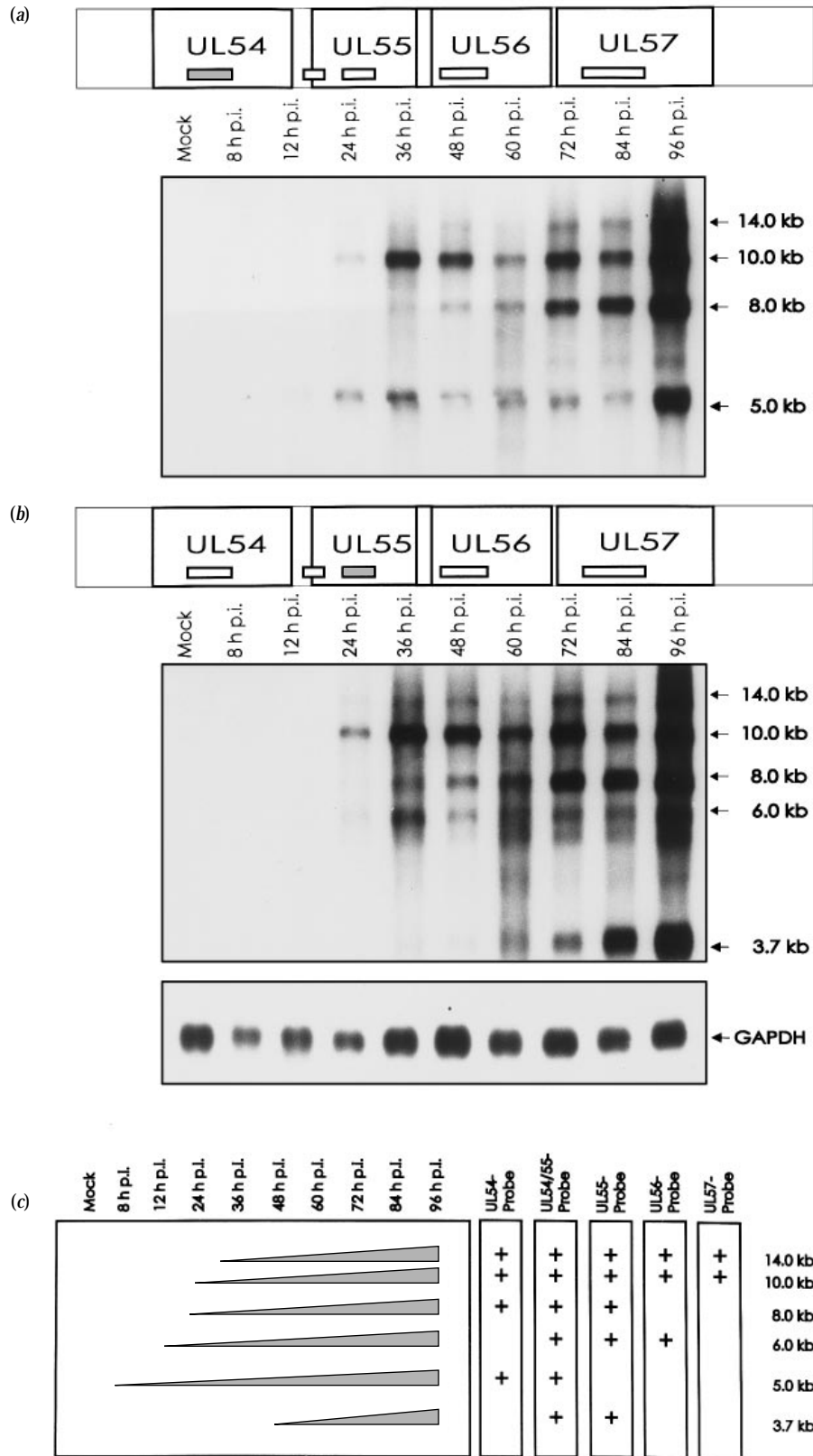


Fig. 2. Time-course of appearance of transcripts during the HCMV infectious cycle. Northern hybridizations were performed with mRNA preparations of mock- and HCMV strain AD169-infected HFF, isolated at 12 h intervals from 8–96 h p.i., and antisense RNA probes specific for (a) ORF UL54 and (b) ORF UL55. The relative positions of the specific probes used are

polyadenylation motifs (AATAAA) located downstream of ORFs UL55 and UL54, respectively. Other potential transcription termination elements (see Fig. 1*a*) were apparently not utilized.

On the basis of this analysis the exact sizes of the observed transcripts [without poly(A)⁺] were calculated: 14 093 nt for the 14 kb transcript representing UL57–UL54; 9916 nt for the 10 kb UL57–UL55 transcript; 9893 nt for the 10 kb UL56–UL54 transcript; 5716 nt for the 6 kb UL56–UL55 transcript; 7647 nt for the 8 kb UL55–UL54 transcript; 3470 nt for the 3.7 kb UL55 transcript; and 4805 nt for the 5 kb UL54 transcript.

Time-course of transcription of ORFs UL54–57 during the infectious cycle

In order to precisely determine whether synthesis of these messengers was initiated at different times during the infectious cycle, parallel cultures of AD169-infected cells were harvested at 12 h intervals starting at 8 h p.i. until 96 h p.i., prior to preparation of mRNAs; mock-infected cultures were used as a control. Preliminary experiments were performed with aliquots of the various mRNA preparations; messengers were approximately quantified using a probe for GAPDH mRNA, which is constitutively expressed in infected cells (see Methods). For consecutive Northern blotting with the gene-specific RNA probes, care was taken to use appropriately adjusted amounts of the mRNA samples for parallel electrophoretic separation (Fig. 2*b*). Hybridization with the UL54 probe revealed the appearance at about 24 h p.i. of high molecular mass messengers of 8 kb, 10 kb and 14 kb (Fig. 2*a*). On the other hand, there was no reaction of messengers of 6 kb and 3.7 kb, as expected (see also Fig. 1*c*). However, an mRNA of 5 kb, most likely representing the monocistronic transcript of the DNA polymerase gene, ORF UL54, was clearly recognized as early as 8–12 h p.i. Using the probe specific for ORF UL55, messengers of 6 kb and 10 kb appeared as early as 24 h p.i. (Fig. 2*b, c*) followed by those of about 8 kb and 14 kb at 24–36 h p.i.; the monocistronic mRNA of 3.7 kb encoded by the gB gene, ORF UL55, was not recognized before 36–48 h p.i. Further Northern blot analyses carried out with probes UL54/55, UL56 and UL57 (data not shown), schematically summarized in Fig. 2*c*, essentially supported the results obtained with probes UL55 and UL54, respectively.

Taken together, our analysis of the time-course of transcription of the gB/POL block genes revealed: (i) approxi-

mately simultaneous appearance early after infection (24 h p.i.) for the doublets of presumably 5' co-terminal transcripts with initiation sites upstream of ORFs UL57 and UL56, respectively, which are terminated either downstream of ORF UL55 or ORF UL54 (mRNAs of 14 kb and 10 kb and those of 10 kb and 6 kb, respectively); (ii) that signals for the monocistronic messengers of the viral DNA polymerase and gB encoded by ORFs UL54 and UL55, respectively, were observed early (8–12 h p.i.) and late (36–48 h p.i.) during the infectious cycle; and (iii) that appearance p.i. differed for the presumably 5' co-terminal transcripts of 3.7 kb and 8 kb, encoded by ORF UL55, and ORFs UL55 and UL54, respectively, with the initiation site upstream of ORF UL55: signals for the bicistronic 8 kb product were obtained early p.i. (12–24 h p.i.), whereas those for the monocistronic 3.7 kb messenger were obtained only late p.i. (about 36 h p.i.). Preparations of mRNA from strain Towne-infected HFF cells or strain AD169-infected U373 cells yielded essentially identical results (data not shown).

Expression of ORF UL55 in the absence of viral DNA synthesis

In order to definitely classify the various transcripts observed as early or late, mRNAs were purified from strain AD169-infected HFF cells that were kept in the presence of phosphonoacetic acid (PAA; 200 µg/ml) or ganciclovir (DPHG; 10 µM) until 48 h p.i. Northern hybridization with probes specific for ORF UL54 and UL55 yielded bands of 5 kb, 6 kb, 8 kb, 10 kb, 14 kb, and of 6 kb, 8 kb, 10 kb, 14 kb (Fig. 3, UL54 and UL55 probes), respectively, and no signal in the range 3.7 kb. This result showed in particular that the 3.7 kb transcript was a late product. Likewise, no signal was obtained using identical preparations from inhibitor-treated infected cells and a specific probe for UL75 (gH gene) which is transcribed only late p.i.; on the other hand, with mRNA from untreated infected cells the expected signal of 2.9 kb was obtained (Fig. 3, UL75 probe). Preparations from strain Towne- or AD169-infected U373 cells yielded identical results; furthermore, none of the probes specific for ORFs UL54, UL55 or UL75 hybridized with mRNA from mock-infected cells (data not shown; see also Fig. 2, mock lanes).

It has been shown previously that the genes for herpesvirus gB homologues belong to the γ_1 class, whose expression is not stringently dependent on viral DNA synthesis (Baradaran *et al.*, 1994). According to this definition, however, the likely HCMV gB messenger of 3.7 kb observed in our experiments

indicated in (a) and (b) by shaded insets in the UL54 and UL55 boxes, respectively. (b) Northern hybridization of identical mRNA preparations with an antisense RNA probe specific for constitutively expressed GAPDH transcripts (see Methods). (c) A schematic presentation of the appearance of gene-specific transcripts during the HCMV infectious cycle is given in the left panel. The signals obtained by Northern blotting at the indicated time points p.i. are schematically summarized for all five gene-specific antisense RNA probes. In the remaining five panels, transcripts recognized by the gene-specific antisense RNA probes (UL54, UL54/55, UL55, UL56 and UL57 probes) are indicated by '+' in the respective columns. The estimated sizes of the specific transcripts are listed on the right.

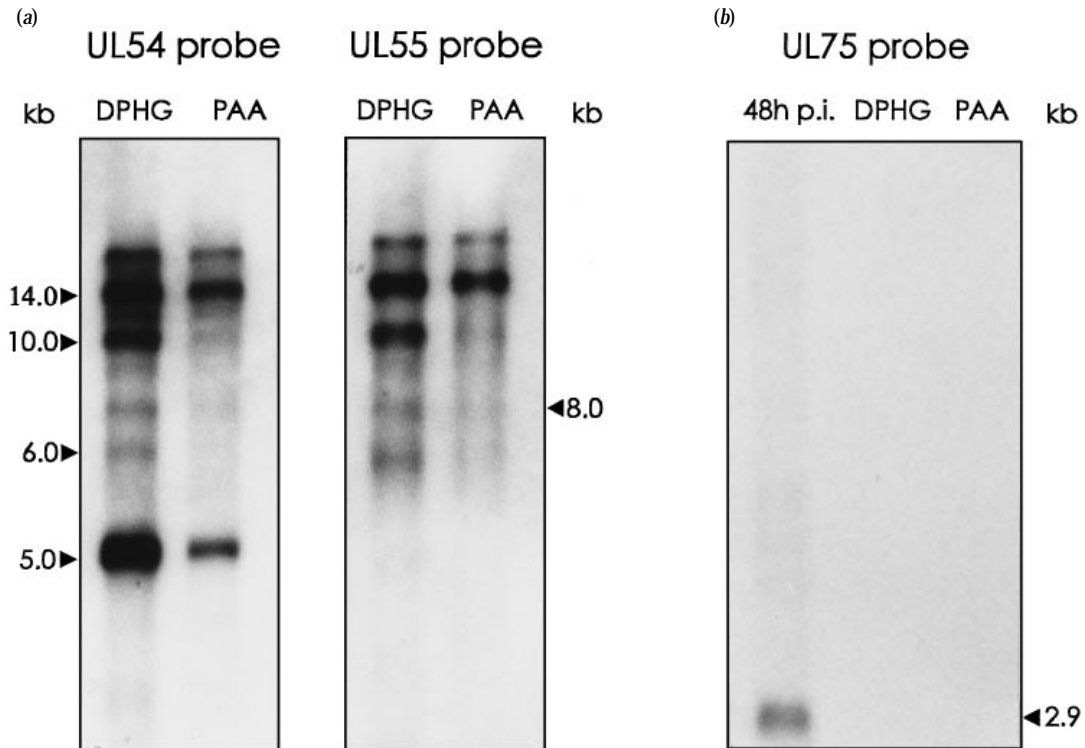


Fig. 3. Identification of mRNAs transcribed from the gB/POL gene cluster in the presence of inhibitors of viral DNA synthesis. (a) Northern hybridizations were performed with mRNA preparations isolated at 48 h p.i. from HCMV strain AD169-infected HFF cells kept in the presence of PAA (200 μ g/ml) or DPHG (10 μ M), and UL54- (UL54 probe) or UL55- (UL55 probe) specific antisense RNA probes. (b) Northern hybridizations of mRNA preparations isolated from untreated (48 h p.i.) and inhibitor-treated HFF cells as in (a) using a gH-specific antisense probe (UL75 probe). The positions of 14 kb, 10 kb, 6 kb and 5 kb messengers are indicated on the left and those of 8 kb and 2.9 kb messengers on the right of the panels.

should be classified as γ_2 , i.e. 'true late', because it was not observed in the presence of inhibitors.

To further elucidate whether a gB-specific polypeptide was translated in the absence of HCMV DNA synthesis, immunoreactions were performed. For immunofluorescence, parallel cover-slip cultures of untreated or inhibitor (PAA; 200 μ g/ml)-treated strain AD169-infected HFF cells (48 h p.i.) were subjected to double staining with a human gB-specific MAb (Ohlin *et al.*, 1993) or gH-specific polyclonal antibody (PAb; Bogner *et al.*, 1992) and MAb against the early HCMV UL112/113-product (M23; Iwayama *et al.*, 1994) (Fig. 4a, a' and b, b'); staining with MAb against the late HCMV antigen MCP was performed as a control (Fig. 4c, d). In untreated infected cells all MAbs specifically recognized the respective antigens (Fig. 4 w/o). In PAA-treated cells, distinct immunolabelling of presumably endoplasmic reticulum (ER)/Golgi structures was observed with gB-specific MAb (Fig. 4b), but not with gH-specific PAb (Fig. 4d); granular nuclear structures were, on the other hand, stained in both samples with MAb against M23 (Fig. 4b', d'). Staining of untreated or inhibitor-treated infected cells with murine gB-specific MAb 27-156 (provided by W. Britt, Birmingham, Alabama, USA; Spaete *et al.*, 1988) alone yielded the same fluorescence pattern as that

obtained with human gB-specific MAb (Fig. 4a, b, data not shown). No specific labelling was obtained in inhibitor-treated cells with MCP-specific MAb (Fig. 4d). Identical observations were obtained with DPHG-treated cover-slip cultures of infected HFF and U373 cells, respectively (data not shown).

For immunoprecipitation, parallel cultures (5×10^6 cells each) of untreated, PAA-treated (200 μ g/ml; 72 h p.i.) or DPHG-treated infected cells were radiolabelled with [35 S]methionine (50 mCi/ml) for 6 h prior to preparation of total cell extracts as described in Methods (Bogner *et al.*, 1992). Immunoprecipitation was performed using a mixture of gB-specific MAb 27-156 and ITC 88 (Ohlin *et al.*, 1993), and the precipitates were analysed by SDS-PAGE (Laemmli, 1970) and fluorography. Mock-infected cells served as a control. Under these conditions, gB-specific polypeptides were precipitated from extracts of untreated infected cells, including the precursor polypeptide of 160 kDa in addition to cleavage products of 90–110 kDa and 55 kDa (Fig. 5 w/o; Britt & Vugler, 1989). Precipitates from inhibitor-treated cells, on the other hand, contained gB precursor polypeptide only at a lower amount than those from untreated cells; furthermore, the inhibitory effect of DPHG appeared to be more pronounced than that of PAA (Fig. 5, PAA and DPHG). These observations

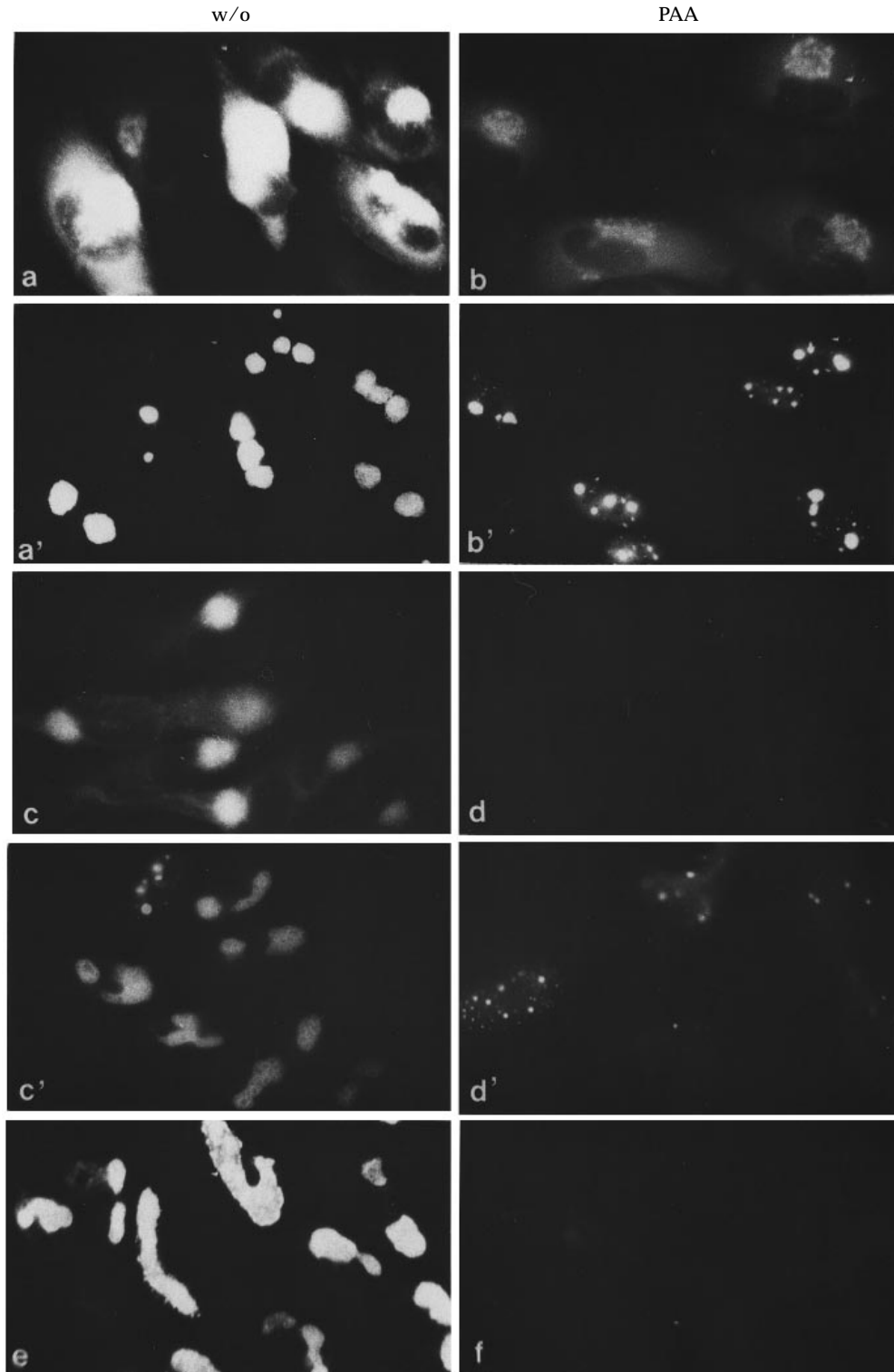


Fig. 4. Recognition by immunofluorescence of HCMV gB in infected HFF cells treated with an inhibitor of viral DNA synthesis. Parallel sets of untreated (w/o) and PAA-treated (200 $\mu\text{g}/\text{ml}$) HCMV strain AD169-infected cover-slip cultures were fixed and permeabilized as described in Methods and double stained with human gB-specific (*a, b*) and mouse anti-M23-specific MAb (*a', b'*), or human gH-specific polyclonal antibody (*c, d*) and mouse anti-M23-specific MAb (*c', d'*). As controls untreated (*e*) and inhibitor-treated (*f*) cover-slip cultures of infected HFF cells were stained with mouse MCP-specific MAb.

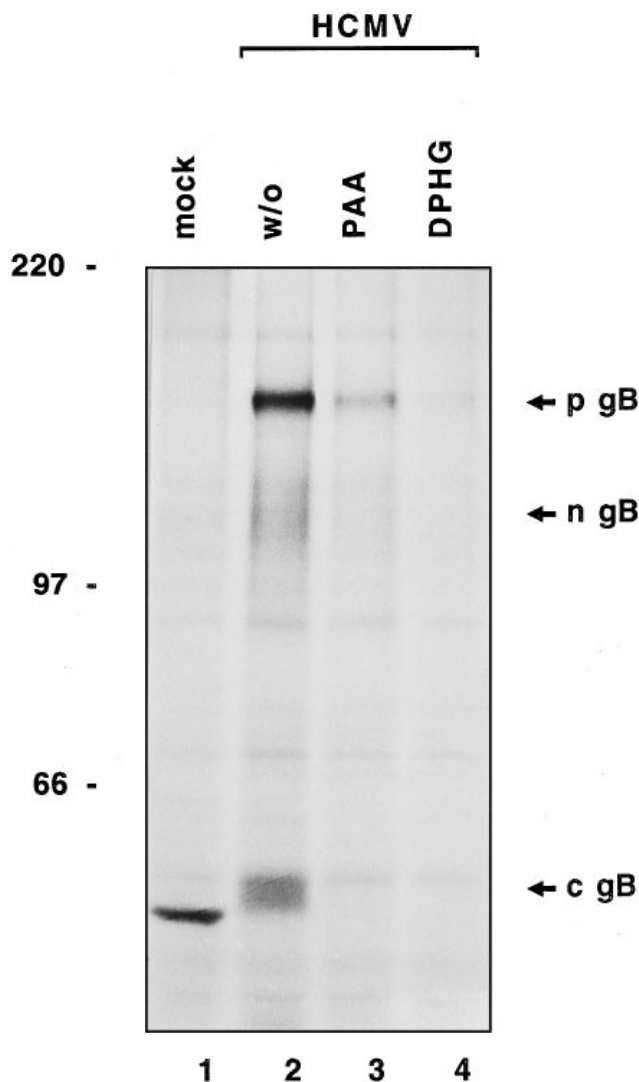


Fig. 5. Recognition by immunoprecipitation of HCMV gB in infected HFF cells treated with inhibitors of viral DNA synthesis. Parallel cultures (5×10^6 cells each) of uninfected (mock) and untreated (w/o), PAA-treated (200 $\mu\text{g}/\text{ml}$) or DPHG-treated (10 μM) HCMV strain AD169-infected HFF cells were radiolabelled as described in the text prior to preparation of extracts which were immunoprecipitated with gB-specific MAbs as described in Methods. Precipitates were analysed by SDS-PAGE and fluorography. The band of 50 kDa in lane 1 (mock) represents an artifactual precipitate of unknown origin. The positions of molecular mass markers (220 kDa myosin, 97 kDa phosphorylase b and 66 kDa bovine serum albumin) are indicated on the left and those of the gB precursor of 160 kDa (p gB) and gB cleavage products of 110 kDa (n gB) and 55 kDa (c gB) by the arrows on the right.

clearly suggest that the HCMV gB homologue is indeed synthesized in the absence of viral DNA synthesis but is not processed by proteolytic cleavage.

Discussion

Previous transcriptional analyses of the HCMV gB/POL block usually dealt only with single ORFs of the gene cluster

and have consistently shown that (i) several signals of variable sizes were recognized in Northern blots by the respective gene-specific probes, suggesting the existence of overlapping 3' or 5' co-terminal transcripts, and (ii) regardless of whether the ORFs encoded either structural or nonstructural viral components, the respective transcripts were, in most of the reports, observed early after infection (Spaete *et al.*, 1988; Kouzarides *et al.*, 1987*a, b*; Mach *et al.*, 1986; Heilbronn *et al.*, 1987; Kemble *et al.*, 1987; Anders & Gibson, 1988; Bogner *et al.*, 1992). Our analysis of the entire gene block presented here using purified mRNAs and highly sensitive as well as specific antisense riboprobes (Engler-Blum *et al.*, 1993) for Northern blotting confirms and extends the notion based on the former reports. A total of seven prominent transcripts was identified, most likely resulting from transcriptional activity driven by four TATA elements (starting at bp 90331, 86130, 83885 and 81041 of the viral genome; Chee *et al.*, 1990) and terminating (at bp 80394 and 76260) downstream of two potential transcriptional stop motifs (bp 80415 and 76329). Regarding for example ORF UL54, the DNA polymerase gene, Kouzarides *et al.* (1987*a*) described mRNAs of 1.6 kb, 4.7 kb and 7.5 kb in addition to larger species which were not clearly resolved. With the exception of the low molecular mass product this compares well to our results demonstrating mRNAs of 5 kb, 8 kb, 10 kb and 14 kb with the probe for UL54 messengers. As for transcription of ORFs UL56 and UL57, the data presented here are largely in line with those reported by Spaete *et al.* (1988) and Kemble *et al.* (1987), respectively, considering that designation of kb sizes for products of high molecular mass is usually an approximation. For transcription initiation directly upstream of ORF UL56 two potential cap sites were observed, one most likely controlled by a prominent TATA element starting at bp 86130 of the viral genomic sequence, and a second one controlled by a potential TATA element starting at bp 86083. Generation of overlapping mRNAs with distinct 5' ends controlled by different promoters in a single transcription unit has also been observed for HCMV ORF UL4 (Chang *et al.*, 1989). It was suggested that heterogeneous 5' ends may play a role in post-transcriptional regulation of gene expression, e.g. at different stages of the infectious cycle.

The transcriptional pattern observed in our experiments with the probe specific for ORF UL55 deserves particular attention. The data obtained partly confirmed previous reports but also differed in essential aspects, e.g. regarding the appearance of gB-specific messages during the HCMV infectious cycle (Spaete *et al.*, 1988; Kouzarides *et al.*, 1987*b*, Mach *et al.*, 1986). It has been a consistent observation that in addition to larger transcripts a prominent HCMV gB-specific mRNA, size 3.7–4 kb, is found in infected cells late p.i. (Kouzarides *et al.*, 1987*b*; Mach *et al.*, 1986). In contrast, Spaete *et al.* (1988) identified this messenger as early as 4 h p.i. as well as in the presence of inhibitors of viral DNA synthesis. Our own results showed again its earliest appearance only by 36 h

p.i. and its complete absence in inhibitor-treated infected HFF and U373 cultures, no matter which virus strain, AD169 or Towne, was used for infection.

Regarding translational expression of HCMV gB at early times p.i. in the absence of viral DNA synthesis, as documented here, it has to be taken into account that all of the probes used also reacted with large early transcripts representative of two to all four ORFs including UL55. Disregarding the hypothetical possibility of internal translation initiation, the presumably bicistronic transcript of about 8 kb is of particular interest in this context since it should contain the ORF UL55 message in the 5' end segment. This gB-specific mRNA of 8 kb was also transcribed in inhibitor-treated cells at reduced levels, and may tentatively be classified as a γ_1 transcript (Baradaran *et al.*, 1994). Interestingly, an early bicistronic gB-specific mRNA of about 7 kb has also been identified for another β -herpesvirus, guinea-pig cytomegalovirus (Schleiss, 1994). Observations comparable to ours, i.e. an early bicistronic messenger originating from the ORFs for gB and for the viral DNA polymerase, and a late monocistronic gB-specific mRNA, have not been described for α -herpesviruses (Pederson *et al.*, 1992). This is not surprising because in this subfamily the ORF for the DNA polymerase is located at the opposite end of the gene cluster adjacent to that of the major single-stranded DNA-binding protein (Albrecht & Fleckenstein, 1990). Regarding HCMV, our observations suggest that gB-related transcriptional and translational activity may be differentially regulated at early and late times p.i.

Our finding that low levels of HCMV gB, but not gH, are expressed in the presence of inhibitors of viral DNA synthesis is intriguing. Herpesvirus gB homologues are generally abundant structural components of the viral envelope which are essential for virus entry and penetration. The known exception is EBV gB, which was identified exclusively in intracellular membranes including the inner nuclear membrane during late lytic infection, but was not incorporated into intra- or extracellular virus particles (Gong & Kieff, 1990). Furthermore, only late mRNAs of 1.8 kb and 3 kb have been identified for EBV gB. To our knowledge there is presently no information available as to whether early gB messengers identified in other herpesvirus systems are indeed also translated (Spaete *et al.*, 1988). As for HCMV, the gB protein expressed in inhibitor-treated cells was not processed by proteolytic cleavage. Even regarding the alternative possibility that the block by high concentrations of inhibitors of DNA synthesis as used here was incomplete, the absence of gB cleavage indicates that cellular transport out of the ER along the exocytic pathway for cleavage by the cellular endoprotease furin in the *trans*-Golgi network (Vey *et al.*, 1995) was inefficient under these conditions; possibly gB transport for efficient proteolytic cleavage is related to the relative abundance of other later viral products. In any case the notion that an 'early' HCMV gB exists needs further investigation, e.g. by characterization of the modifications of this product and eukaryotic

expression of appropriate constructs containing ORFs UL55 and UL56.

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