JOURNAL OF VIROLOGY, June 1993, p. 3515–3527
0022-538X/93/063515-13$02.00/0

Vol. 67, No. 6

Characterization and Temporal Regulation of mRNAs Encoded by Vaccinia Virus Intermediate-Stage Genes

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Received 21 December 1992/Accepted 17 March 1993

The steady-state levels of mRNAs encoded by three intermediate-stage genes of vaccinia virus, A1L, A2L, and G8R, were compared with those encoded by well-characterized early- and late-stage genes. After synchronous infection of HeLa cells, the early mRNA was detected within 20 min and peaked at about 100 min; all three intermediate mRNAs were detected at 100 min and peaked at about 120 min; and the late mRNA was detected at 140 min and increased thereafter. Upon reaching maximum levels, the early and intermediate mRNAs declined at rates consistent with half-lives of about 30 min, providing the basis for rapid changes in gene expression. Intermediate mRNA was not detected when viral DNA synthesis was prevented, whereas its accumulation was enhanced by blocking translation after removal of the replication inhibitor. The 5′ ends of the mRNAs initiated within a TAAAT or TAAAAT sequence in the coding DNA strand but contained a poly(A) leader of up to 30 additional bases. Diffuse bands of A1L and G8R RNA, equal to and longer than the coding region, were resolved by agarose gel electrophoresis, suggesting preferred sites of 3′-end formation that did not correlate with early gene termination signals. The *cis*-regulatory sequences were investigated by constructing recombinant viruses containing mutated intermediate promoters preceding the β-galactosidase reporter gene. The effects of mutations on expression were similar to those previously obtained by transfection studies (C. J. Baldick, Jr., J. G. Keck, and B. Moss, J. Virol. 66:4710–4719, 1992), providing further evidence for functional core, spacer, and initiator regions. In addition, an up-regulated bifunctional early/intermediate promoter was created by making four single-base substitutions in the G8R promoter.

Vaccinia virus, the best-studied member of the poxvirus family, contains approximately 200 genes that are closely spaced along its 200,000-bp linear double-stranded DNA genome (19). Viral replication occurs in the cytoplasm of the host cell, consistent with observations that many proteins necessary for RNA and DNA synthesis are virus encoded. The viral genes can be grouped into three classes, early, intermediate, and late, according to the stage of infection in which they are activated (reviewed in references 32 and 33). Soon after entering the cell, a complete transcription system within the virus particle produces early mRNAs. This system includes a multisubunit DNA-dependent RNA polymerase, a heterodimeric transcription factor, and enzymes for capping and polyadenylation of the nascent transcripts. The translation products of the early mRNAs include enzymes and factors for DNA replication and intermediate gene expression (23, 45). The latter consist of RNA polymerase, capping enzyme, and at least one other transcription factor (43, 44). Following DNA replication, intermediate mRNAs are made. Their translation products include three *trans* activators of late genes (23, 51, 52). The late mRNAs encode virion proteins, including the two subunits of the early transcription factor. In this manner, each gene class is activated by specific transcription factors synthesized during the prior phase of gene expression.

A large number of early genes have been identified by their expression under conditions in which DNA replication or protein synthesis is prevented. The early gene transcriptional regulatory elements of some were found to reside within a region extending about 30 bp upstream of the mRNA start site (11, 48). An extensive mutational analysis of one early promoter defined a critical A+T-rich sequence between −13 and −28 (14). Initiation occurs at a purine residue (+1) located downstream from this promoter element. Discrete 3′ ends are formed by transcription termination which occurs about 50 bp downstream of the sequence UUUUUNU, usually located near the end of the protein-coding region of the mRNA (39, 42, 53). Analyses of steady-state mRNA and pulse-labeled polypeptides have shown that early mRNA levels and protein synthesis peak between 1 and 3 h postinfection (hpi) and then decline (12, 29, 34, 38).

Although the existence of an intermediate and late class of post-DNA replication proteins (34, 38) and mRNAs (47) had been suggested, specific intermediate genes were only recently identified (23, 45). The five known intermediate genes were distinguished from late genes by their expression when transfected into vaccinia virus-infected cells in the presence of an inhibitor of DNA replication. Mutational analyses of promoters for several genes demonstrated that the regulatory sequences were contained within a 30-bp region upstream of the RNA start site (4, 20). A detailed analysis of the G8R, A1L, and A2L promoters revealed a core element between −26 and −13 and a TAAA initiator element between −1 and +3 as the critical regions for promoter function (4). Although the A+T content of the intermediate core element is similar to that of the early promoter, the specific sequence requirements are different. mRNA synthesis initiated within a TAAAT motif in the A1L promoter and in a TAAAAT motif in the A2L and G8R promoters. However, the 5′ and 3′ ends of intermediate transcripts have not been characterized, nor has an analysis of the temporal steady-state levels of specific intermediate mRNAs been reported.

The regulatory elements for late gene promoters are rather

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short, requiring only 16 to 27 bp of DNA upstream of the RNA start site (5, 10, 49). The important promoter elements appear to be a TAAAT motif at the transcriptional start site and upstream T residues (15). mRNAs transcribed from typical late genes have a 5′ poly(A) leader of up to 40 bases (2, 6, 26, 40). In the few early promoters in which transcription initiation occurred within a TAAAT sequence, the mRNAs generated contained a non-template-encoded poly(A) leader sequence of 4 to 15 nucleotides (1, 3, 21). The poly(A) is believed to form by RNA polymerase slippage following initiation in the TAAAT element (15, 16). Late transcription does not respond to the early termination signal, and long mRNA molecules with extreme 3′-end heterogeneity result (13, 17, 25, 28, 36). Late mRNA and proteins are detected at about 3 to 4 hpi, and synthesis continues throughout the replication cycle (34, 38, 47).

To better understand the regulation of vaccinia virus gene expression, we have investigated the synthesis and structure of intermediate mRNAs. The intermediate genes A1L, A2L, and G8R were selected for study because their products are of particular importance as late gene *trans* activators and their promoters have been well characterized. Here, we report the results of a steady-state analysis of these intermediate mRNAs during the course of a synchronous virus infection, the presence of poly(A) leaders at the 5′ ends of intermediate mRNAs, evidence for length heterogeneity of intermediate transcripts with possible preferred regions of 3′-end formation, and the effects of promoter mutations on reporter gene expression in recombinant viruses.

(This work was carried out by C. Baldick, Jr., in partial fulfillment of the Johns Hopkins University requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Cells and virus. Human 293 cells, thymidine kinase (TK)-negative 143 cells, and monkey BSC-1 cells were propagated in Eagle's minimal essential medium (Quality Biologicals, Inc.) supplemented with 10% fetal calf serum in 5% CO_2 at 37°C. Monkey CV-1 cells were maintained as described above but with Dulbecco's modified minimal essential medium. HeLa S3 spinner cells were propagated in minimal essential spinner medium (Quality Biologicals, Inc.) supplemented with 5% horse serum at 37°C. Vaccinia virus and the recombinant vaccinia viruses were derived from the WR strain (ATCC VR1354). Titers of purified and crude virus stocks were determined by plaque assay on BSC-1 monolayers. Purified virus was sonicated for 30 s before use. Aliquots of crude virus were incubated with an equal volume of trypsin (0.25 mg/ml) for 30 min at 37°C prior to sonication.

Recombinant virus construction. Plasmid pLacZ (4) contains the *Escherichia coli lacZ* gene positioned just downstream of unique restriction endonuclease sites that were used to clone viral promoter fragments. These promoter fragments contained the transcriptional and translational start sites of the intermediate A1L, A2L, or G8R gene plus 300 bp of upstream sequence. The procedures used to clone the fragments into pLacZ have been described in detail elsewhere (4). Digestion of each plasmid with *Sal*I-*Hin*dIII liberated a 3.5-kbp fragment containing the intermediate promoter-*lacZ* cassette, which was subsequently treated with DNA polymerase I (Klenow fragment) to create blunt ends. Plasmid pGS-8 (27), which contains the vaccinia virus TK gene on a 2.6-kbp viral DNA fragment, was linearized at a unique site within the TK gene by *Eco*RI digestion and treated with the Klenow fragment of DNA polymerase. Each blunt-ended intermediate promoter-*lacZ* fragment was then ligated to pGS-8, and plasmids which contained the *lacZ* gene in the opposite transcriptional orientation to the disrupted TK gene were selected and amplified. The sequences of the 300-bp promoter fragment and the 5′ end of the *lacZ* gene were then confirmed by using the Sequenase kit (United States Biochemical). These vectors were used to insert the intermediate promoter-*lacZ* cassette into the TK region of the vaccinia virus genome by homologous recombination (27). Recombinant viral plaques were identified by staining cell monolayers with 0.3 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and blue plaques were picked as described previously (8). After three rounds of plaque purification, each of three recombinant viruses was amplified and purified by two sucrose gradient sedimentations (22). A virus containing the *lacZ* gene, but lacking the intermediate promoter, was also constructed and used as a background control.

For experiments using mutated G8R intermediate promoters, a 30-bp promoter fragment was cloned into the vector pMJ35 (14) such that β-galactosidase expression from the *lacZ* gene was controlled by the intermediate promoter. These constructs were used to create recombinant viruses with the intermediate promoter-*lacZ* cassette inserted in the TK locus in the same manner as described above. Methods for synthesis and cloning of the mutated promoters have been described in detail elsewhere (4). Recombinant viruses were identified as described above but maintained as crude stocks consisting of infected HeLa cell lysates. A recombinant virus lacking a promoter was constructed and used as a background control in all experiments in which β-galactosidase or mRNA levels were analyzed.

RNA purification. Total cellular RNA was purified from uninfected or infected cells by the acid guanidinium thiocyanate-phenol-chloroform method, using RNAzol (TM Cinna Scientific, Inc.) as described by the manufacturer. Approximately 2 μg of RNA per 10^5 cells was obtained and stored at a concentration of 5 to 10 mg/ml in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) at −70°C. For some experiments, further purification of mRNA by using the Poly(A) Quik mRNA purification kit (Stratagene) was performed as instructed by the manufacturer. Eluted poly(A)$^+$ RNA was ethanol precipitated and stored at a concentration of 2 mg/ml in TE at −70°C.

Steady-state RNA analysis. HeLa suspension cells (10^7/ml) were infected in a spinner culture at a multiplicity of infection (MOI) of 20. After a 30-min adsorption period, the cell culture was diluted to 5×10^5 cells per ml by the addition of spinner medium containing 5% horse serum. An aliquot of 10^7 infected HeLa cells was removed from the spinner culture at the indicated times and centrifuged at 2,000 rpm for 3 min. The medium was aspirated, and the cell pellet was then immediately placed on dry ice. RNA was extracted as described above. In the experiments indicated, DNA replication was blocked by addition of hydroxyurea (HU) to a final concentration of 10 mM at the end of the adsorption period. The drug was washed out (to allow DNA replication to begin) 3 hpi by centrifuging the cells at 2,000 rpm for 5 min and resuspending them in an equal volume of medium lacking HU. In some experiments, protein synthesis was blocked by addition of cycloheximide to a final concentration of 100 μg/ml. RNA expression directed by mutated intermediate promoters was analyzed by infecting monolayers of 293 cells in 12-well Costar tissue culture trays (10^6 cells per well) at an MOI of 10. RNA was extracted 18 hpi as described above.

Templates for the in vitro synthesis of antisense RNA probes consisted of target DNA sequences fused downstream of the bacteriophage T7 ϕ10 promoter. Templates for the synthesis of A1L, A2L, and G8R riboprobes were produced by hybridizing complementary primers approximately 200 bp upstream and 300 bp downstream of the transcriptional start site of the appropriate gene present on subcloned viral DNA. The downstream primer contained additional sequences comprising a T7 promoter. The target sequence was amplified by 15 polymerase chain reaction cycles. The template for the vaccinia virus growth factor (VGF; C11R) gene consisted of an 638-bp *Hin*cII-*Acc*I fragment, containing the VGF transcriptional start site, cloned into the in vitro RNA synthesis vector pGEM-3Z (Promega). After digestion with *Eco*RI, runoff transcription generated a 678-base probe which would protect 316 bases of the 5′-end of the VGF transcript. The template for the 11-kDa structural protein gene (F17R) consisted of a 670-bp *Bam*HI-*Hin*dIII fragment cloned into pGEM-4Z (Promega). After digestion with *Bam*HI, runoff transcription generated a 682-base probe which would protect 126 bases of the 5′ end of the 11-kDa transcript. Individual templates for each mutated intermediate G8R promoter were made by the polymerase chain reaction technique described above except that the downstream primer was located 200 bp from the transcriptional start site. T7 RNA polymerase was used to produce [α-^{32}P]UTP (3,000 Ci/mmol, 10 mCi/ml)-labeled RNA probes (as described by Promega) complementary to the appropriate mRNA.

Total cell RNA (10 μg) was hybridized overnight at 42°C to a molar excess of labeled complementary RNA probes in 30 μl of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl–1 mM EDTA–80% deionized formamide. Digestion was performed in 300 μl of 10 mM Tris-HCl (pH 7.5)–5 mM EDTA–0.3 M NaCl with RNase A (4 μg/ml) and RNase T_1 (10 U/ml) at room temperature for 1 h. Protected probe fragments were analyzed by electrophoresis on a 6% polyacrylamide–8 M urea gel and visualized by autoradiography. A Molecular Dynamics phosphoimager or a Betagen Betascope 603 blot analyzer was used for quantitation.

Primer extension analysis. Total cell RNA (20 μg) harvested 2 hpi was annealed to the appropriate 5′-end-labeled primer (1 pmol) as in the RNase protection experiments described above. Primers were gel-purified oligonucleotides complementary to A1L, A2L, or G8R mRNA between +36 and +65, relative to the transcriptional start site. Hybridization was carried out at 42°C overnight. Following ethanol precipitation, the pellet was resuspended in 10 μl of 50 mM Tris-HCl (pH 8.3)–75 mM KCl–5 mM $MgCl_2$ containing 200 U of Moloney murine leukemia virus reverse transcriptase (BRL-Life Technologies, Inc.). This reaction mixture was divided into five aliquots of 2 μl each, to which 2 μl of deoxynucleotide or dideoxynucleotide mixture was added. The deoxynucleotide solution consisted of 2.5 mM each dATP, dCTP, dGTP, and dTTP. The dideoxynucleotide mixtures were as follows: the ddATP mixture consisted of 1 mM each dCTP, dGTP, and dTTP, 0.5 mM dATP, and 2.5 mM ddATP; the ddCTP mixture consisted of 1 mM each dATP, dGTP, and dTTP, 0.5 mM dCTP, and 2.5 mM ddCTP; the ddGTP mixture consisted of 1 mM each dATP, dCTP, and dTTP, 0.5 mM dGTP, and 2.5 mM ddGTP; the ddTTP mixture consisted of 1 mM each dATP, dGTP, and dCTP, 0.5 mM dTTP, and 2.5 mM ddTTP. Samples were incubated at 42°C for 30 min, and the extended products were analyzed by electrophoresis on a 6% polyacrylamide–8 M urea gel and visualized by autoradiography.

Nuclease S1 analysis. Total cell RNA (20 μg) harvested 2 hpi or yeast tRNA (20 μg) was annealed to the appropriate 5′-end-labeled probe (0.01 pmol) as in the RNase protection assay described above. After overnight hybridization at room temperature, samples were placed on ice for 5 min. Digestion was performed in 300 μl of 50 mM sodium acetate (pH 4.6)–4.5 mM $ZnSO_4$–0.25 M NaCl–20 μg of salmon sperm DNA per ml containing 1,000 U of nuclease S1 (BRL-Life Technologies, Inc.) per ml at 15°C for 1 h. Protected probe fragments were analyzed by electrophoresis on a 6% polyacrylamide–8 M urea gel and visualized by autoradiography. The wild-type probe was complementary to the coding-strand genomic sequence of the G8R gene between −20 and +65, relative to the transcriptional start site. The poly(T) probe was identical to the wild-type probe from +1 to +65 but contained 20 thymidine residues from −1 to −20 in place of the genomic sequence. Both probes were purified by gel electrophoresis.

Northern (RNA) blot analysis. Poly(A)$^+$ RNA (2 μg) either from uninfected HeLa spinner cells or from infected cells harvested 2 hpi was separated on a 1.5% formaldehyde–agarose gel in 40 mM morpholine propanesulfonic acid (MOPS; pH 7.0)–10 mM sodium acetate–1 mM EDTA. A mixture of single-stranded RNAs (BRL-Life Technologies, Inc.), visible after ethidium bromide staining, was used as molecular weight standards. Fractionated mRNA was then transferred to a Nytran membrane (Schleicher & Schuell) overnight by capillary action as described by the manufacturer. Prehybridization and hybridization were done in Quikhyb rapid hybridization solution (Stratagene) for 1 h each at 55°C. The hybridization solution contained an internally labeled riboprobe complementary to the intermediate gene mRNA. The probes were synthesized in vitro by a method similar to the one used to make the probes for the RNase protection. Oligonucleotide primers were hybridized to each intermediate gene subcloned on a plasmid, and a 200-bp region in the middle of each open reading frame was amplified by the polymerase chain reaction. The downstream primer in each set contained additional sequences specifying the T7 promoter. Templates were transcribed in vitro with T7 RNA polymerase to make 200-base riboprobes as described for the RNase protection above.

β-Galactosidase measurements. Time course experiments measuring β-galactosidase synthesis regulated by the wild-type intermediate promoters were performed by infecting HeLa suspension cells (10^7 cells per ml) with the appropriate recombinant vaccinia virus at an MOI of 20. After a 30-min adsorption period, the cell culture was diluted to 5×10^5 cells per ml with spinner medium containing 5% horse serum. An aliquot of 5×10^5 cells was removed at the indicated time points and centrifuged at 2,000 rpm for 3 min, and the cell pellet was resuspended in 200 μl of phosphate-buffered saline. Cells were lysed by three freeze-thaw cycles and then centrifuged to remove cellular debris. A colorimetric assay was used to measure β-galactosidase activity present in cell extracts (30). The amount of protein, determined by using the Bio-Rad protein quantitation reagent, was then used to normalize the β-galactosidase measurements as previously described (4). β-Galactosidase activity values (typically 1% or less) obtained from cells infected with a recombinant virus lacking a viral promoter controlling *lacZ* were subtracted as background.

Experiments measuring the β-galactosidase activity regulated by mutated intermediate promoters were performed by

infecting 293 cell monolayers (10^6 cells per well in 12-well trays) with recombinant vaccinia viruses at an MOI of 10. After 30 min, the inoculum was removed and replaced with minimal essential medium containing 5% fetal calf serum. In the indicated experiments, cytosine arabinoside (AraC) was added to the medium to a final concentration of 40 μg/ml. Cells were harvested 18 hpi and resuspended in 0.5 ml of phosphate-buffered saline. Cells were then lysed and assayed for β-galactosidase, and the background was subtracted as described above. Infections were done in duplicate wells, and the average β-galactosidase activity values are reported (standard deviations are indicated when greater than 10%).

β-Galactosidase activity values reported for transfected plasmids containing intermediate promoters were obtained from previous work (4). Promoter mutant S-G8R was characterized by transfection in this study, using methods identical to those described in reference 4. Transfections were done in duplicate, and the average β-galactosidase values were reported (standard deviations were less than 10%).

RESULTS

Steady-state intermediate mRNA levels. An RNase protection assay was used to analyze the steady-state levels of the mRNAs transcribed from the three intermediate genes, A1L, A2L, and G8R. Internally labeled complementary RNA probes specific for the 5′ end of the A1L, A2L, and G8R intermediate transcripts were synthesized in vitro by using T7 RNA polymerase. Analogous probes for the well-characterized VGF early mRNA and the 11-kDa polypeptide late mRNA were made so that the transcripts of all three vaccinia virus gene classes could be compared simultaneously. In initial experiments, HeLa spinner cells were infected with vaccinia virus at an MOI of 20 and aliquots of cells were removed at 2, 4, 6, 8, and 22 hpi. In all experiments, 0 time refers to the time of addition of virus and not to the end of the 30-min adsorption period. Equal amounts of total mRNA prepared from infected and uninfected cells were then incubated with the early, intermediate, and late riboprobes. Control experiments demonstrated that all probes were in molar excess relative to the target RNA. After RNase digestion, the protected probe fragments were run on a polyacrylamide gel and visualized by autoradiography.

RNA from each time point was analyzed simultaneously for the presence of VGF (early) and 11-kDa (late) transcripts (Fig. 1A). VGF mRNA was abundant at 2 hpi, but only a small percentage remained by 4 hpi. The mRNA was nearly gone by 6 hpi and barely detectable by 22 hpi. mRNA derived from the 11-kDa gene first appeared at 4 hpi, and the steady-state levels remained constant or increased slightly throughout the time course of the experiment. RNAs from the same time points were also analyzed for the presence of the intermediate transcripts. The results indicated (Fig. 1B) that the G8R mRNA had accumulated by 2 hpi, declined steadily through 8 hpi, and increased again by 22 hpi. None of the probes hybridized to RNA isolated from uninfected HeLa cells.

Both the VGF and G8R transcripts were present at 2 hpi (Fig. 1A and B), the earliest time examined. However, previous work had demonstrated that A1L, A2L, and G8R were not transcribed if DNA replication was blocked with the inhibitor AraC (23). To further distinguish the early and intermediate genes, RNA was isolated from HeLa cells infected for 4 h in either the presence or absence of cycloheximide, an inhibitor of protein synthesis. Blocking early protein synthesis not only prevents DNA replication but has the added effect of superinducing early mRNA transcription (12, 13, 50), thus increasing the sensitivity for detecting early genes. RNase protection assays failed to detect a transcript from the A1L, A2L, or G8R gene when the cells were infected in the presence of cycloheximide (Fig. 1C). In contrast, large amounts of the VGF transcript were visible in the presence of cycloheximide. Therefore, transcription of the A1L, A2L, and G8R genes requires de novo protein synthesis, unlike the situation for early genes. Additional bands were detected in the A1L and A2L samples at 4 hpi in the absence of the drug. The presence of late genes (including the strong P4b protein gene) immediately upstream of A1L and A2L presumably generates read-through transcripts that result in this type of artifact, which has also been noted by other investigators (23, 28, 29, 31, 47). The additional bands were not detected at 2 hpi, consistent with this interpretation (data not shown).

The data presented above indicated that transcription of all three classes of vaccinia virus genes occurred within 4 h after infection. To derive a detailed temporal picture of the steady-state levels of intermediate mRNA, infected HeLa spinner cells were harvested at 20-min intervals for a period of 4 h. RNase protection using probes for all three classes of RNAs yielded the results shown in Fig. 2. VGF early mRNA was detected at 20 min after infection, peaked at about 100 min, and then abruptly declined. Each of the three intermediate gene mRNAs was detected first at 100 min after infection, peaked at about 120 min, and then declined. A minor band just above the major A1L species increased in intensity during this period, probably as a result of late read-through transcripts. The 11-kDa late mRNA was barely detected at 140 min after infection and gradually increased through the 4-h period. Because of differences in the amounts of the various mRNAs, the autoradiographic film exposure times were varied as indicated in the legend to Fig. 2. Therefore, the levels of the different mRNAs cannot be compared by visual inspection. Radioactive protected probe fragments were quantitated directly from the gels by using a Betagen Betascope blot analyzer. The values were then normalized for the number of uridine residues in the probe fragments. When plotted together, the results illustrate the quantitative relationship between early, intermediate, and late steady-state mRNA levels over a 4-h time period (Fig. 3). The amount of VGF mRNA probably reflects the high multiplicity and synchronicity of infection in HeLa spinner cells. As accumulation of the VGF early mRNA peaked, intermediate mRNAs from the A1L, A2L, and G8R genes appeared. The 11-kDa late mRNA level rose as the intermediate levels peaked, and by 4 hpi the late mRNA was the most abundant species. The steady-state level of the 11-kDa mRNA reached a maximum by 6 hpi and persisted at high levels for at least 22 h (Fig. 1A). Seven to ten times more G8R RNA than A1L or A2L mRNA was present at the 2-hpi peak. This difference was also reflected in the amount of protein expressed from a reporter gene controlled by these intermediate gene promoters (see below). Nevertheless, the kinetics of synthesis and degradation of all three intermediate mRNAs were very similar.

Effects of inhibitors of DNA replication and protein synthesis on mRNA levels. Previous studies showed that inhibitors of DNA replication inhibit intermediate as well as late gene transcription (23, 45). This effect on the transcription of the I3L intermediate gene was reversed by washing out the replication inhibitor at the same time that an inhibitor of protein synthesis was added (45). By contrast, postreplica-

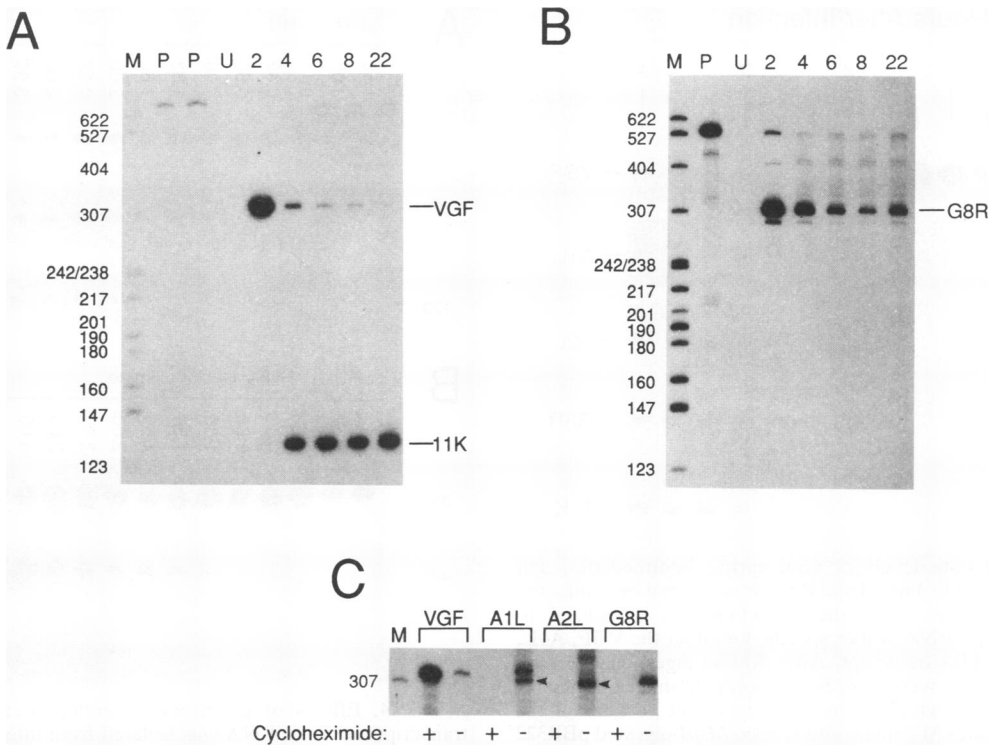

FIG. 1. Steady-state levels of viral early-, intermediate-, and late-stage mRNAs. (A) Analysis of early and late mRNAs. Total RNA was isolated from uninfected or infected HeLa cells at various times after infection and hybridized simultaneously to ^{32}P-labeled antisense RNA probes specific for either the VGF or 11-kDa transcript. After RNase digestion, the protected probe fragments were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane M contains end-labeled *Msp*I-digested pBR322 fragment markers (the sizes in base pairs are indicated at the left). The second and third lanes labeled P contain the undigested VGF and 11-kDa probes, respectively. The remaining lanes contain samples derived from uninfected cells (U) or samples derived from cells infected for 2 through 22 hpi. The expected sizes of the probe fragments protected by the VGF and 11-kDa transcripts are indicated at the right. (B) Analysis of intermediate mRNA. RNA samples were derived as in panel A but were hybridized to an RNA probe specific for the G8R transcript. Samples were analyzed as in panel A. (C) Effect of protein synthesis inhibition on early and intermediate transcription. Total RNA was isolated from infected HeLa cells in either the presence (+) or absence (−) of cycloheximide at 4 hpi and hybridized to ^{32}P-labeled antisense RNA probes specific for either the VGF, A1L, A2L, or G8R transcript. Samples were analyzed as in panel A; only the significant portion of the gel is shown. The expected sizes of the probe fragments protected by the A1L and A2L intermediate transcripts are indicated by the arrowheads.

tive protein synthesis was required for transcription of a late gene. These results are consistent with the encoding of intermediate and late transcription factors by early and intermediate genes, respectively. We wished to extend these results by using an intermediate gene, G8R, that encodes a known late transcription factor and by making a detailed kinetic analysis. HeLa spinner cells were infected with vaccinia virus in the presence of HU, a reversible inhibitor of DNA replication. After 3 h, the cells were resuspended in medium lacking HU and in the absence (Fig. 4A) or presence (Fig. 4B) of cycloheximide, an inhibitor of protein synthesis. Cells were collected at 20-min intervals, and RNA was isolated and analyzed by RNase protection, using probes specific for the 5′ end of the VGF early, the G8R intermediate, or the 11-kDa late transcript. Even after 3 h in the presence of HU, only the early mRNA was produced (Fig. 4). Upon removal of the HU, intermediate and late mRNAs appeared in succession (Fig. 4A). Within 40 min, the G8R transcript could be detected, probably reflecting the accumulation of early proteins required for DNA replication and intermediate gene transcription during the incubation with HU. The accumulation of transcription factors also may account for the persistence of relatively high G8R mRNA levels over the next few hours. The interval of 80 to 100 min between the detection of intermediate and late transcripts, however, was similar to that which occurred during a normal infection, consistent with the requirement for de novo synthesis of intermediate proteins.

Addition of cycloheximide at the time of removal of HU did not affect the onset of G8R transcription but greatly increased the amount of the mRNA that accumulated (Fig. 4B). Moreover, no late mRNA was detected under these conditions, consistent with the requirement for intermediate proteins for late transcription.

Analysis of the 5′ ends of the intermediate mRNAs. Previous nuclease S1 protection experiments (23, 46) and the RNase protection studies described above suggested that the transcriptional start sites of the A1L, A2L, and G8R genes occurred near or within TAAAT of TAAAAT sequences preceding the open reading frames. The latter procedures, however, cannot detect the presence of the 30- to 40-nucleotide 5′ poly(A) leaders on late transcripts, which typically initiate within TAAAT sequences, or the shorter 5′ poly(A) leaders on those early mRNAs that initiate within a TAAAT sequence. To characterize the 5′ ends of the intermediate mRNAs, we performed primer extension analyses. RNA was harvested from cells at 2 hpi, which represents the peak of intermediate mRNA synthesis and precedes the

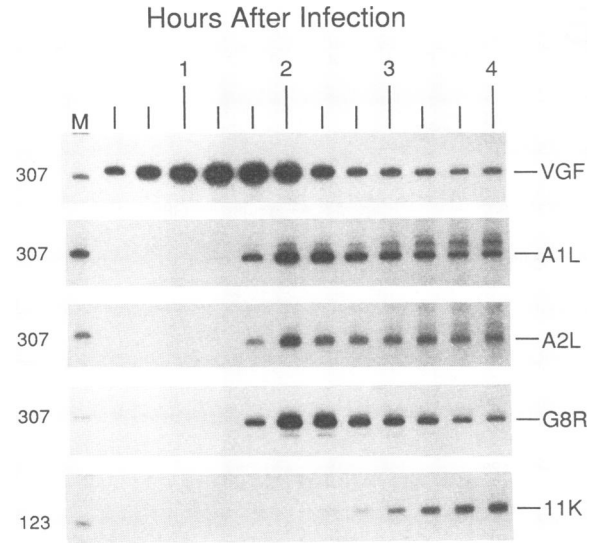

FIG. 2. Steady-state levels of viral early-, intermediate-, and late-stage mRNAs up to 4 hpi. Total RNA was isolated from infected HeLa cells at various times after infection and hybridized to ^{32}P-labeled antisense RNA probes specific for either the VGF, A1L, A2L, G8R, or 11-kDa transcript. After RNase digestion, the protected probe fragments were analyzed by polyacrylamide gel electrophoresis and autoradiography. Only the significant portions of the gels are shown. Lanes M contain end-labeled *Msp*I-digested pBR322 fragment markers (the sizes in base pairs are indicated at the left). The remaining lanes contain samples derived from infected cells every 20 min through 4 hpi. The expected sizes of the protected probe fragments are indicated at the right. Autoradiographic exposure times: VGF and 11-kDa transcript, 45 min; A1L and A2L, 90 min; G8R, 20 min.

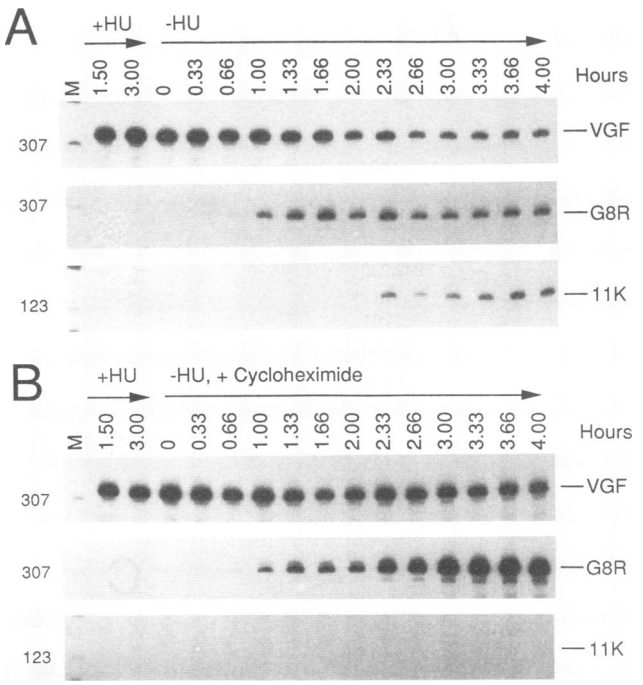

FIG. 4. Effects of inhibitors on early, intermediate, and late transcription. Total RNA was isolated from infected HeLa cells at various times after infection and hybridized to ^{32}P-labeled antisense RNA probes specific for either the VGF, G8R, or 11-kDa transcript. After RNase digestion, the protected probe fragments were analyzed by polyacrylamide gel electrophoresis and autoradiography. Only the significant portions of the gels are shown. Lanes M contain end-labeled *Msp*I-digested pBR322 fragment markers (the sizes in base pairs are indicated at the left). (A) Infected HeLa cells were incubated for 3 h in medium containing HU and then for 4 h in medium lacking the drug. RNA was obtained from cells collected at 1.5 and 3.0 hpi in the presence of HU, immediately after the HU was removed (0), and at 20-min intervals through the subsequent 4 h. (B) Infected HeLa cells were incubated for 3 h in medium containing HU and then for 4 h in medium in which the HU was replaced with cycloheximide. The times used to obtain RNA were the same as in panel A.

appearance of late mRNAs. We especially wanted to avoid the period of late transcription, since late mRNAs are known to be heterogeneous in length and can overlap genes located downstream. Using the A1L primer, mRNA molecules extending up to 30 bases past the transcriptional start site (+1, based on previous mapping [46]) were observed (Fig. 5A). In addition, the sequence of the mRNA (represented by the first four lanes) correlated with that of the DNA up to the position marked as +1 (Fig. 5B). The bands upstream of the +1 suggested a heterogeneous-length leader varying from 3 to 30 nucleotides in length. Primer extension using the A2L or G8R primer gave similar results but indicated that the leaders were only 4 to 15 nucleotides long.

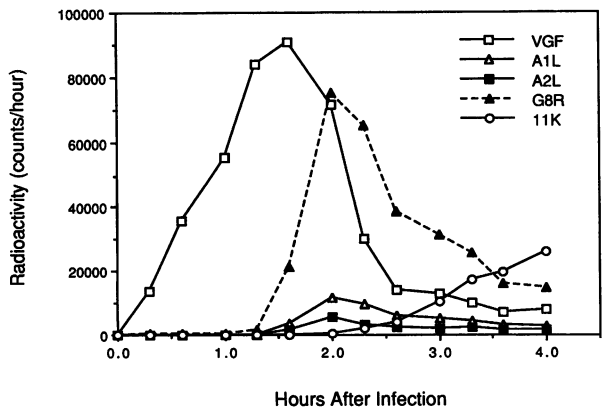

FIG. 3. Quantitation of early-, intermediate-, and late-stage mRNAs. The dried gels from Fig. 2 were scanned with a Betagen Betascope blot analyzer. The counts for the protected probe fragments from each time point were then normalized for the number of uridines.

To determine whether the additional sequences present on the 5′ ends of the intermediate mRNAs were A residues, a nuclease S1 analysis of G8R mRNA was performed with two kinds of probes. The first probe (Fig. 6B, wild-type probe) was a 5′-end-labeled oligonucleotide designed to be complementary to the G8R coding strand, between −20 and +65 on the genomic DNA sequence. RNA harvested 2 hpi that was hybridized to this probe and digested with nuclease S1 yielded a short array of bands, of which the most predominant was 65 nucleotides long (Fig. 6A, lane 6), corresponding to the first A in the TAAAAT motif of the G8R promoter. The second probe was an oligonucleotide identical to the wild-type probe from +1 to +65 but containing only T residues from −1 to −20 [Fig. 6B, poly(T) probe]. Hybridization of this probe to the same RNA (Fig. 6A, lane 7) yielded protected bands 1 to 11 bases longer than that

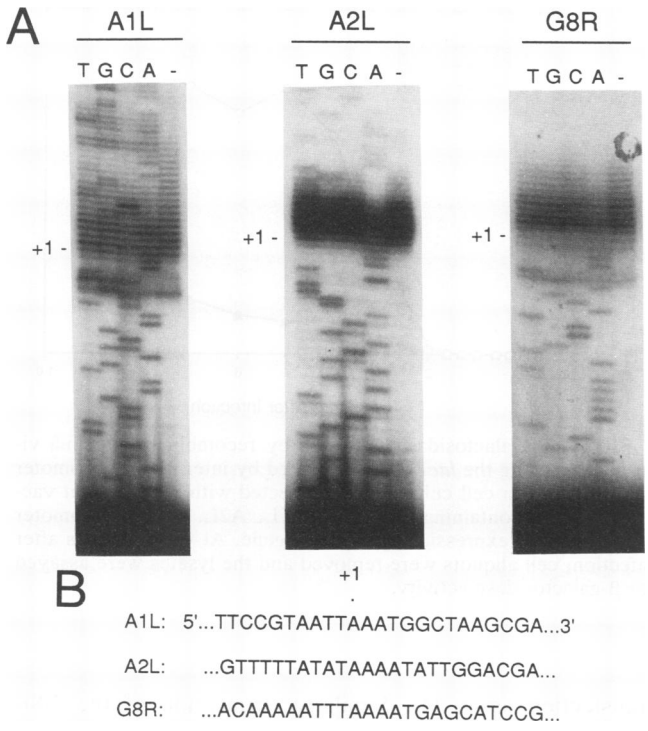

FIG. 5. (A) Primer extension analysis of the 5′ ends of the intermediate transcripts. Total RNA was isolated from infected HeLa cells 2 hpi and hybridized to a 5′-end-labeled oligonucleotide complementary to sequences from +36 to +65 of either the A1L, A2L, or G8R mRNA. The primer was elongated with reverse transcriptase in the presence of ddA (lane T), ddC (lane G), ddG (lane C), or ddT (lane A) or without dideoxynucleotides (lane −). Reactions were analyzed by polyacrylamide gel electrophoresis and autoradiography. The transcriptional start sites mapped by nuclease S1 analysis (23, 46) are indicated at the left as +1. (B) DNA sequences corresponding to the regions surrounding the A1L, A2L, and G8R transcriptional start sites.

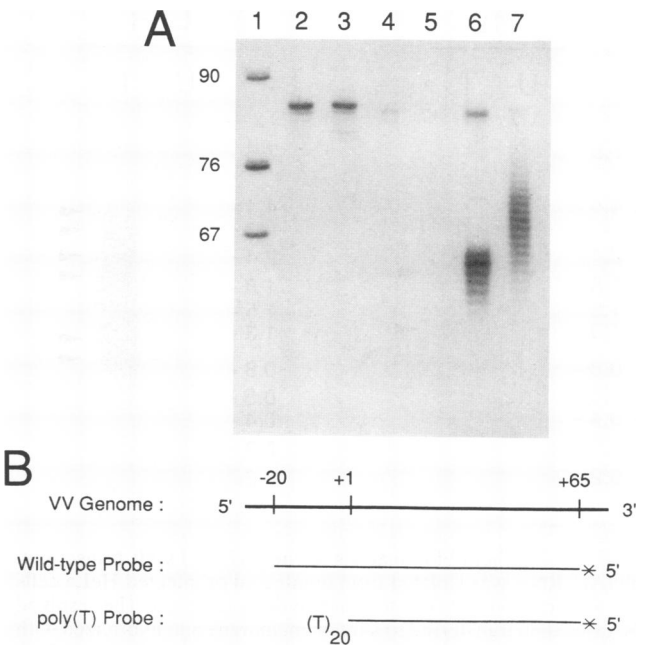

FIG. 6. (A) Nuclease S1 analysis of the 5′ end of the G8R transcripts. Total RNA was isolated from uninfected or infected HeLa cells 2 hpi and hybridized to one of two 5′-end-labeled oligonucleotide probes. The wild-type probe was complementary to sequences from −20 to +65 of the coding strand of the G8R gene. The poly(T) probe was identical to the wild-type probe from +1 to +65 but contained 20 thymidine residues from −1 to −20 (see panel B). Following nuclease S1 digestion, the protected probe fragments were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane 1 contains end-labeled *Msp*I-digested pBR322 fragment markers (the sizes in base pairs are indicated at the left). Lanes 2 and 3 contain the undigested wild-type and poly(T) probes, respectively. Uninfected cell RNA (lane 4) or infected cell RNA (lane 6) was hybridized to the wild-type probe and digested with nuclease S1. Uninfected (lane 5) or infected (lane 7) cell RNA was hybridized to the poly(T) probe and digested with nuclease S1. (B) Diagrams of the vaccinia virus (VV) genomic region around the G8R transcriptional start site (+1) and of the corresponding wild-type and poly(T) probes. The asterisk designates the 5-end label of each probe.

obtained with the wild-type probe. No bands were detected after digestion when either probe was incubated with RNA from uninfected cells (Fig. 6A, lanes 4 and 5). These results, together with the primer extension analysis, indicated the presence of a short poly(A) leader on the G8R transcripts. Although this nuclease S1 analysis with a poly(T) probe was not performed on the A1L or A2L RNA, we believe that the additional leader sequences present are also A residues.

Northern blot analysis of intermediate mRNAs. Transcription of early genes results in mRNAs with defined 3′ ends, and Northern blots usually result in distinct bands consistent with the size of the open reading frame. By contrast, typical late gene transcripts have long and heterogeneous 3′ ends. When Northern blotting was performed, this heterogeneity led to a smear resulting from mRNA molecules from 1 to more than 6 kb in length (13, 17, 25, 28, 36). To determine whether intermediate gene transcripts have defined 3′ ends, Northern blot analyses were done. Poly(A)$^+$ RNA isolated from HeLa cells 2 hpi was size fractionated on a formaldehyde-agarose gel and transfered to a nylon membrane. In vitro-synthesized, internally labeled RNA probes complementary to a 200-bp region in the middle of the A1L, A2L, or G8R coding region were used to detect the transcripts. No hybridization to uninfected cell mRNA was detected with any of the probes; however, two faint bands (marked by arrowheads) were detected with the infected cell mRNA on the A1L blot (Fig. 7). In addition, the A1L probe hybridized to a smear of viral mRNAs ranging in size from about 0.4 to 4.5 kb, although the lower-molecular-weight RNA is not visible in the autoradiographic reproduction. Similarly, the G8R probe detected a series of six diffuse viral RNA bands ranging in size from about 0.8 to 4.5 kb. The background smears visible with both probes were similar to those seen on Northern blots of late transcripts. However, the mRNA used for the blot was obtained from HeLa cells 2 hpi; therefore, the smear should not be a consequence of late transcription. Northern blotting of early poly(A)$^+$ mRNA harvested from HeLa cells 4 hpi in the presence of cycloheximide was also performed with the G8R probe. None of the bands seen with the 2-hpi RNA were visible (data not shown), indicating that the bands were not early transcripts. Since only a single transcriptional start site was detected for the G8R gene, the range of transcript sizes is most likely due to 3′-end heterogeneity. The lower range of the RNA detected with the A1L or G8R probe is approximately the minimum size necessary for the mRNA to include the entire

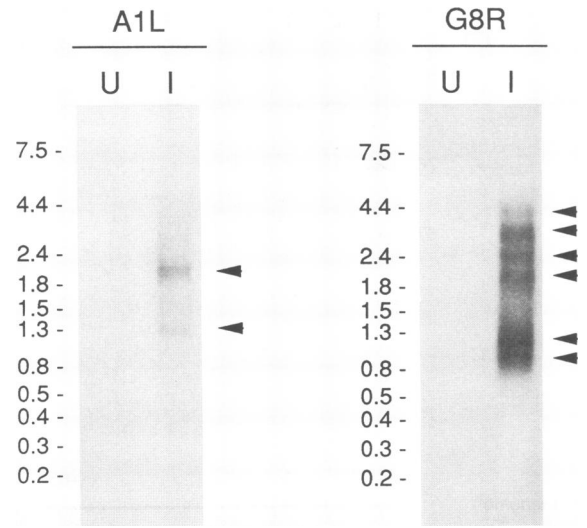

FIG. 7. Northern blot analysis of the A1L and G8R transcripts. Poly(A)$^+$ RNA was isolated from uninfected or infected HeLa cells 2 hpi and fractionated on a formaldehyde-agarose gel. The resolved RNA was then transferred to a nylon membrane and hybridized with a ^{32}P-labeled antisense RNA probe specific for mRNAs which contain the A1L or G8R open reading frame. Lanes U contain uninfected cell RNA; lanes I contain infected cell RNA. Sizes of marker RNAs in kilobases are shown at the left. Arrowheads at the right indicate the series of RNA species detected.

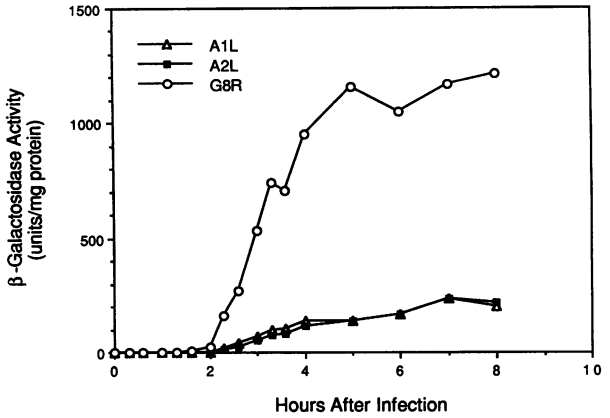

FIG. 8. β-Galactosidase synthesis by recombinant vaccinia viruses containing the *lacZ* gene regulated by intermediate promoter elements. HeLa cell cultures were infected with recombinant vaccinia viruses containing either the A1L, A2L, or G8R promoter controlling the expression of the *lacZ* gene. At various times after infection, cell aliquots were removed and the lysates were assayed for β-galactosidase activity.

protein-coding region. Results of the A2L Northern blot were difficult to interpret because of the low amount of mRNA and the resulting weak signal (data not shown). Efforts to map the precise 3′ ends of the intermediate G8R transcripts by nuclease S1 analysis were unsuccessful, perhaps because of 3′-end microheterogeneity.

Regulation of β-galactosidase synthesis by intermediate promoters. Recombinant vaccinia viruses which contained either the A1L, A2L, or G8R intermediate promoter controlling the expression of the *lacZ* gene were constructed. The plasmids containing the individual promoter-*lacZ* cassettes were recombined into the TK locus of the viral genome. Initial constructs contained intermediate promoter fragments which extended 300 bp upstream of A1L, A2L, or G8R intermediate gene transcriptional start site. To confirm that the translocated promoters were regulated in an authentic temporal manner, separate HeLa spinner cell cultures were infected with each recombinant virus. At various times, the cells were harvested and the β-galactosidase activity in the cell lysates was measured. β-Galactosidase activity was detected at or slightly before 2 hpi in each case (Fig. 8). This time was consistent with the analysis of the A1L, A2L, and G8R transcripts (Fig. 2). In addition, the relative levels of β-galactosidase produced by each recombinant correlated with the relative mRNA levels (Fig. 3) produced by the natural genes. Control infections in the presence of AraC resulted in no β-galactosidase expression (data not shown), as would be expected for an intermediate promoter. The translocated intermediate promoters attached to reporter genes therefore appear to regulate expression in the same manner as do their natural counterparts.

Analysis of intermediate promoter mutations. Deletion analysis had previously shown that a region extending 30 bp upstream of the RNA start site was sufficient for the A1L, A2L, and G8R intermediate gene promoters to function in a transfection assay (4). Similarly, truncation of the G8R promoter fragment in the recombinant virus described above from 300 to 30 bp had no effect on β-galactosidase expression (data not shown). Point mutations of the G8R intermediate promoter, which were characterized by transfection, defined two domains (−26 to −13 and −1 to +3) critical for promoter function (4). Of the set of promoters with point mutations regulating the *lacZ* gene, nine with G substitutions were placed in recombinant viruses. Five of these mutations (−26G, −22G, −19G, −18G, and −13G) were in the upstream core domain. Three mutations (−1G, +1G, and +3G) were in the initiator domain. All eight of these substitutions had been previously demonstrated to drastically reduce the function of the G8R promoter in transfection assays. Another single-point substitution mutant was constructed (−7G) in the spacer region of the promoter, which had been shown by transfection to have no effect on promoter activity. We also made a recombinant virus (S-G8R) containing four nucleotide substitutions in the G8R promoter core domain which we predicted would increase expression twofold (4). Control recombinant viruses which had a wild-type G8R promoter or no promoter controlling the *lacZ* gene were also made. Monolayers of 293 cells were infected with each recombinant virus, and β-galactosidase activity was measured at 18 hpi. These values were compared with levels previously reported by transfection (4) (Table 1). Most of the mutations had similar effects on the regulation of *lacZ* expression when present in a recombinant virus or transfected plasmid. Substitutions at positions −26 and −22, however, had a more detrimental effect on the G8R promoter when analyzed by transfection. The promoter in construct S-G8R, containing four point mutations, was tested for activity by plasmid transfection in the presence of AraC and by recombinant virus infection in the absence of the inhibitor. The results indicated that while the S-G8R promoter produced the predicted amount of β-galactosidase by transfection (approximately two times the wild-type value), greater than 5.5 times more β-galactosidase was produced by the recombinant virus. The almost threefold difference between the activities seen by transfection and recombinant

TABLE 1. Effect of promoter mutations on β-galactosidase activity

Promoter	β-Galactosidase activity	
	Plasmid transfection[a]	Recombinant vaccinia virus[b]
Wild-type G8R	1.00	1.00
None	0.00	0.00
−26G	0.06	0.18
−22G	0.01	0.24
−19G	0.00	0.01
−18G	0.00	0.00
−13G	0.09	0.12
−1G	0.16	0.05
+1G	0.03	0.01
+3G	0.00	0.00
−7G	1.14	1.08
S-G8R	2.04	5.59

[a] All values (except for S-G8R) were derived from reference 4. The S-G8R construct was characterized in this report, and the activity value was determined as in reference 4. Activities are normalized to that of the wild-type promoter (set at 1.00).

[b] Normalized to total cell protein and to the activity of the wild-type promoter (set at 1.00). Determinations were made in duplicate, and the standard deviations were less than 10%.

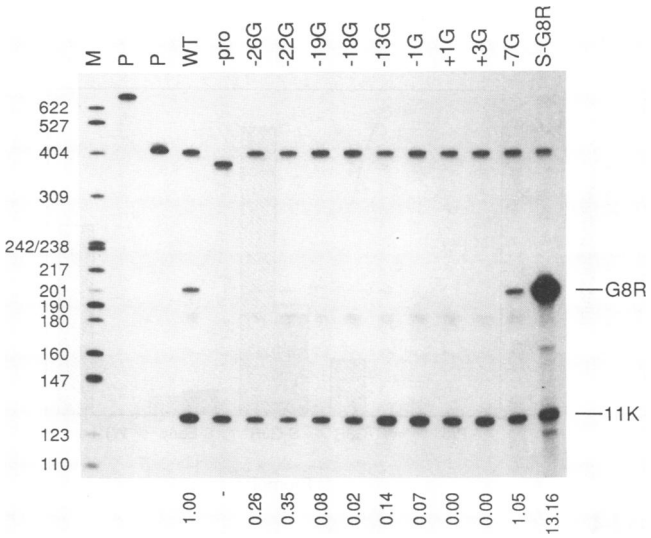

FIG. 9. Analysis of mRNA synthesized by G8R promoter mutants. Total RNA was isolated from 293 cells infected with vaccinia virus recombinants containing mutations of the G8R promoter regulating the *lacZ* gene. The RNA was hybridized simultaneously to ^{32}P-labeled antisense RNA probes specific for each promoter mutant and to the 11-kDa late gene transcripts (which served as an internal control). After RNase digestion, the protected RNA was analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane M contains end-labeled *Msp*I-digested pBR322 fragment markers (the sizes in base pairs are indicated at the left). The second and third lanes labeled P contain the undigested 11-kDa and wild-type G8R probes, respectively. The remaining lanes contain protected RNA samples derived from cells infected with the recombinant viruses containing the wild-type (WT) G8R promoter, no promoter (-pro), single G substitutions at the indicated nucleotide number, or the up-regulated G8R promoter (S-G8R). Numbers below the lanes indicate the quantitated values of the protected wild-type or mutant G8R probe fragments determined with a Molecular Dynamics Phosphoimager, normalized first to the values of the internal 11-kDa fragments and then to the wild-type value. The expected sizes of the probe fragments protected by the G8R and 11-kDa transcripts are indicated at the right.

virus was not, however, unexpected, as will be explained below.

In addition to monitoring promoter function by quantitating β-galactosidase activity, we analyzed the mRNA level produced by each mutant. RNA was harvested from 293 cells infected with the wild-type G8R promoter recombinant virus, the recombinant virus lacking an intermediate promoter, or one of the recombinant virus promoter mutants. Riboprobes specific for each construct were used in an RNase protection assay (Fig. 9). As an internal control, RNA transcripts from the natural 11-kDa gene were analyzed simultaneously. Results were quantitated with a Phosphoimager, and transcripts originating from the G8R promoter or one of the promoter mutants were then normalized by comparison with the 11-kDa transcripts. These values were then standardized to wild-type promoter, which was assigned a value of 1, and the results are displayed below the lanes in Fig. 9. Relative mRNA levels correlated well with the β-galactosidase activities measured for each construct (Table 1), with the exception of the S-G8R mutant. This mutant produced about two times more mRNA than expected from the β-galactosidase assays. Although not every nucleotide substitution within the G8R intermediate promoter analyzed by transfection was also tested by construction of the corresponding recombinant virus, the results of the subset tested here are consistent with the existence of the two critical domains and the spacer domain in the G8R intermediate promoter.

Construction of a biphasic early/intermediate promoter. The up-regulated intermediate promoter (S-G8R) described above was constructed by combining four base substitutions in the core domain of the G8R promoter. Substitutions of an A residue at −23 and a G residue at −20 were predicted to give the twofold increase in promoter strength that was subsequently verified by the intermediate promoter-specific transfection assay (Table 1). On the basis of previous transfection assays (4), substitutions of A residues at positions −16 and −12 were predicted to be silent and have no effect on intermediate promoter activity. These mutations were included because they made the S-G8R promoter's core domain similar in sequence to a strong early promoter critical domain (vMJ343 [14]) (Fig. 10B). Knowing that the only requirement for early promoters is an upstream critical domain of acceptable sequence and a purine residue at +1 (14), we expected the S-G8R promoter to function as both an intermediate and an early promoter. Our transfection analysis verified that the S-G8R promoter was indeed a strong intermediate promoter (Table 1). However, transfection assays are not suitable for measuring early promoter function (4, 9). To test the potential early activity of the promoter, the S-G8R recombinant virus was used to infect 293 cell monolayers in either the presence or absence of AraC. When DNA replication is blocked with AraC, only early viral genes are expressed. Recombinant viruses containing either no promoter, the wild-type G8R intermediate promoter (WT-G8R), the strong synthetic early promoter (S-Early), or the 11-kDa late promoter (WT-Late), all driving the *lacZ* gene, were tested in parallel infections. The amounts of β-galactosidase produced by the recombinant viruses under each condition were then determined. WT-G8R expressed β-galactosidase only after DNA replication, as would be expected for an intermediate promoter (Fig. 10A). However, the S-G8R promoter recombinant virus

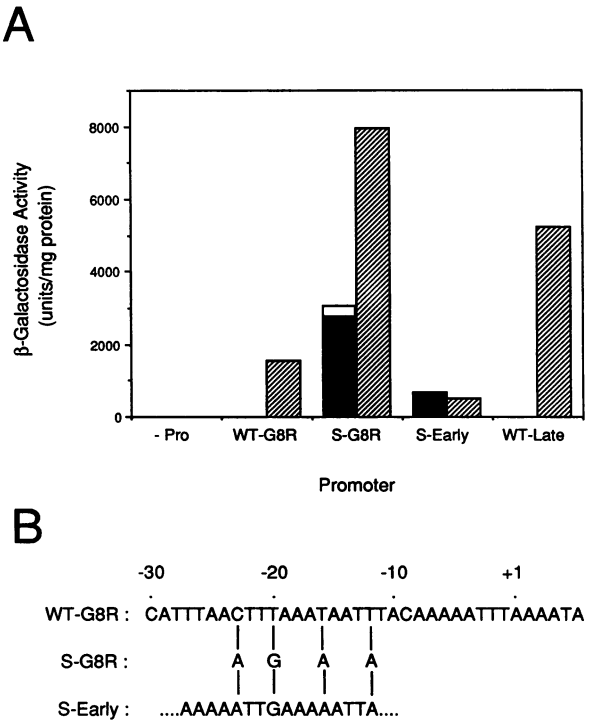

FIG. 10. (A) Pre-and post-DNA replication activity of the up-regulated G8R promoter. β-Galactosidase activity was measured from monolayers of 293 cells that were infected with recombinant vaccinia viruses that contained either the wild-type G8R promoter (WT-G8R), the up-regulated G8R promoter (S-G8R), the strong synthetic early promoter (S-Early; vMJ343 [14]), the 11-kDa late promoter (WT-Late), or no promoter (-Pro) regulating the *lacZ* gene. Filled and hatched bars are infections done in the presence and absence of AraC, respectively. Standard deviations (when >10%) are represented as open bars. (B) Sequence comparisons of the WT-G8R promoter, the S-G8R promoter, and the S-Early promoter. The region between −30 and +6 (+1 indicating the transcriptional start site) of the WT-G8R promoter is depicted. Below the WT-G8R promoter sequence are the substitutions made to create the up-regulated G8R promoter. The critical region of the strong synthetic early promoter is shown below, aligned with the S-G8R and the WT-G8R promoters.

expressed β-galactosidase in the presence and absence of AraC, indicating that it was also an early promoter. Interestingly, the S-G8R promoter was three times stronger than the up-regulated synthetic vaccinia virus early promoter (S-Early) in the presence of AraC. Lack of activity from the construct with the late 11-kDa promoter (WT-Late) in the presence of AraC confirmed that the drug was effective in blocking DNA replication. The increased amount of β-galactosidase produced by the S-G8R promoter in the absence of AraC, compared with the WT-G8R promoter, was probably due to the cumulative effects of the early and up-regulated intermediate expression. The S-G8R promoter can therefore be defined as a bifunctional early/intermediate promoter.

DISCUSSION

Although the time course of transcription of early- and late-stage genes had been determined in some detail (12, 29, 47), the temporal synthesis of the intermediate-stage mRNAs was largely inferred from transfection and inhibitor experiments (23, 45). In the present study, we used RNase protection assays to directly quantitate the steady-state levels of mRNAs transcribed from three intermediate genes, A1L, A2L, and G8R, and compared them with levels of well-characterized early and late mRNAs.

To obtain synchronicity, concentrated suspensions of HeLa cells were infected with a viral multiplicity of 20. RNA was then prepared from cells that were harvested at 20-min intervals. Transcription of an early gene (represented in our studies by the VGF gene) was detected at the first time point. The steady-state level of early mRNA peaked at about 100 min and then declined rapidly so that only 10% of the maximal value was present at 220 min. As the early mRNA level peaked, transcription of the A1L, A2L, and G8R intermediate genes began. These mRNAs rapidly reached peak levels at about 120 min and then exhibited a biphasic decline. Although there was 7 to 10 times more mRNA synthesized from the G8R gene than from the A1L or A2L gene, the apparent kinetics of synthesis and degradation displayed by the three intermediate mRNAs were almost identical. Finally, a late gene transcript (represented in our studies by the 11-kDa protein gene) was detected at 140 min, immediately following the peak in intermediate mRNA levels. Steady-state levels of the late transcript rose until about 6 hpi and remained relatively constant through 22 hpi. Interestingly, the absolute amount of VGF mRNA at its peak was greater than that of any of the three intermediate mRNAs or the strongly expressed 11-kDa late mRNA. Evidently, the large amount of late viral proteins reflects the long period of late mRNA synthesis rather than abundance. The sequential onset of early, intermediate, and late mRNA synthesis is consistent with earlier amino acid pulse-labeling studies (34, 38) and with cascade models of gene regulation (18, 23, 45). This model states that early mRNAs encode intermediate transcription factors, intermediate mRNAs encode late transcription factors, and late mRNAs encode early transcription factors.

The kinetics of mRNA synthesis following reversal of a DNA replication block also support the cascade model. Vos and Stunnenberg had found that inhibition of de novo protein synthesis following DNA replication prevented transcription of the 11-kDa late gene but not the I3L intermediate gene (45). We extended this observation by performing a detailed temporal analysis of the steady-state levels of early, intermediate, and late mRNAs under conditions that restricted DNA or protein synthesis. HeLa cells were infected in the presence of HU, a DNA synthesis inhibitor, to allow early proteins to accumulate. The DNA replication block was then lifted, and in the presence or absence of cycloheximide, the subsequent mRNA levels were measured over time. As expected, G8R mRNA appeared soon after HU removal even in the absence of further protein synthesis. By contrast, there was about an 80- to 100-min interval between intermediate and late transcription during which protein synthesis was required. Evidently, the intermediate transcription factors were made in the presence of HU, thereby allowing intermediate mRNAs to be formed after DNA replication occurred. The translation of this mRNA into the G8R and other late transcription factors, however, was necessary for late mRNA synthesis. Other in vivo studies using inducer-dependent (54) and temperature-sensitive (7) mutants of late transcription factors also support the cascade model. Under conditions in which cells were infected in the presence of HU followed by cycloheximide addition at the time of removal of the HU, the steady-state levels of VGF mRNA persisted at a high level over a period of several hours. This effect may be similar to that of cycloheximide added at the

start of infection, which greatly increases early mRNA synthesis apparently by blocking the disassembly of the virion transcription system (50). The same conditions of HU reversal and cycloheximide addition also lead to increasing steady-state levels of G8R mRNA. There are several possible explanations for this phenomenon. One is that excess intermediate transcription factors were produced during the prolonged period of early gene expression. Another is the absence of late transcription factors which ordinarily compete with intermediate factors for RNA polymerase or other general transcription components. Intermediate or late proteins also could be involved more directly in repression of intermediate transcription or in destabilizing intermediate transcripts.

The regulation of viral gene expression depends on the rapid rate of mRNA degradation as well as on sequential transcription mechanisms. The short half-lives of viral mRNAs were first noted by Oda and Joklik (35) and Sebring and Salzman (41). We can estimate from the rapid changes in steady-state levels of the early VGF mRNA that its half-life cannot be longer than 30 min. This number is shorter than the 2 h determined for bulk early mRNAs (35, 41) or for transcripts encoding the DNA polymerase (29). Whether the different values reflect a particular instability of the VGF mRNA or the tissue culture cells and infection conditions used is unclear. The steady-state levels of the three intermediate mRNAs first declined abruptly, consistent with a half-life of about 30 min, but then declined more slowly. The slow decline phase and the persistence of low levels of intermediate mRNAs may result from continued synthesis and degradation late in infection. Since the A1L, A2L, and G8R genes have TAAAT or TAAAAT initiation elements, it is possible that they function as weak late promoters as well as intermediate promoters.

Transcripts originating from the A1L, A2L, and G8R intermediate genes all appeared to have poly(A) leaders on their 5′ ends, presumably as a result of a slippage of the RNA polymerase as suggested for late transcripts (15, 16). Intermediate promoters that have TAAAAT motifs at the transcriptional start site (A2L and G8R) generated mRNAs with leaders of between 4 and 15 residues, similar to the number of adenosine residues seen on early transcripts that have poly(A) leaders (1, 3, 21). mRNAs from the A1L intermediate gene had leaders of 3 to 30 residues, similar to late transcripts (2, 6, 40). The A1L promoter, like late promoters, has a TAAAT motif at the start site. Additional intermediate gene promoters need to be studied to determine whether the longer poly(A) leaders on the A1L transcripts correlate with the TAAAT motif and whether the shorter poly(A) leaders of the A2L and G8R transcripts correlate with the TAAAAT motif. While the TAAAT motif is an essential initiator element of late promoters (15), TAAA is the minimal initiator sequence necessary for intermediate promoters to function (4). Whether TAAA followed by nucleotides other than T or AT can generate intermediate mRNA with poly(A) leaders has not been determined. Intermediate transcripts from the I3L and I8R genes reportedly lack poly(A) leaders (45). However, neither promoter contains a TAAA(A)T motif at the reported RNA start site. Early promoters require no such initiator motif, and its presence merely results in addition of a poly(A) leader without affecting the quantity of mRNA transcribed (21).

The nature of the 3′ ends of the intermediate transcripts is not yet clear. Northern blot analyses of oligo(dT)-selected A1L and G8R transcripts resulted in a series of two (A1L) or six (G8R) diffuse bands that range in size from about 0.8 to 4.5 kb. A background smear, reminiscent of Northern blots of late mRNA (13, 25, 28), was visible over the entire range. The sizes of the bands on the Northern blot do not correspond to mRNAs that would be predicted if transcription terminated in response to the early termination signals (TTTTTTNT) that are present in the DNA sequence, consistent with in vitro studies (44). We are unable at this time to correlate any specific DNA sequence with the bands on the Northern blot. The size of the lowest-molecular-weight RNA detected was approximately equal to the minimum size necessary to contain the entire A1L or G8R protein-coding region. In this respect, the Northern blots of late transcripts are similar in that the smears normally detected begin at about the minimum size needed for the mRNA to encode the particular protein (13). Whether the bands of intermediate mRNA seen were a result of transcription termination, or posttranscriptional processing as occurs with the ATI mRNA of cowpox virus (3a, 37), remains to be determined.

The functional domains of intermediate promoters were previously identified by linker scanning (20) and single-nucleotide substitution mutagenesis (4). The latter more detailed study, however, was carried out by transfection of plasmids in which mutated promoters were linked to a reporter gene. We have evaluated the conclusions derived from the transient assays by making recombinant viruses containing representative promoter mutations. β-Galactosidase activity and mRNA directed by these recombinant viruses correlated with the transfection data, consistent with the presence of the core, spacer, and initiator elements in the genomic G8R promoter. A mutant promoter (S-G8R) was up-regulated for intermediate promoter activity and also designed to have strong early promoter activity by making four nucleotide substitutions in the core region. This is the first known example of an early/intermediate promoter. As the S-G8R promoter is as active as the late 11-kDa gene promoter, it may be useful for expression vectors. Two other examples of biphasic poxvirus promoters have been reported. The cowpox virus early 38-kDa gene promoter was modified to create a TAAAT motif at the transcriptional start site, resulting in transcription both before and after DNA replication (21). Similarly, a fowlpox virus promoter that is active both before and after DNA replication has been identified (24). In neither of these cases was an experimental distinction made between intermediate and late transcription, leaving the possibility that these promoters are early/intermediate or early/late in function. Indeed, sequences upstream of these RNA start sites resemble an intermediate core element. The determination of whether an intermediate promoter also has late promoter activity is technically difficult at this time.

The similarities of the core sequences of early and intermediate promoters and of the initiator sequences of intermediate and late promoters seem consistent with evolutionary relationships between them. The pre- and postreplicative transcription systems of primitive poxviruses might have recognized one promoter type, perhaps resembling our early/intermediate promoter. There would be an obvious advantage, however, in regulating gene expression. The early or prereplicative system may have adapted primarily to the core element. At this stage in evolution, poxviruses may have had only a single postreplicative transcription system resembling the intermediate class. The emergence of a late postreplicative regulatory system, adapted to the initiator element, may have provided an additional level of control. The cataloging of homologous genes of different poxviruses with respect to early, intermediate, and late promoters may

provide further insight into the evolution of the transcription system.

ACKNOWLEDGMENTS

We thank Jerry Sisler for synthetic oligonucleotides, John Coligan for use of the Phosphoimager, and Elizabeth Wolffe and James Keck for critical review of the manuscript.

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