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Regulation of Murine Cytomegalovirus Gene Expression
I. Transcription During Productive Infection

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Murine cytomegalovirus RNA synthesis in productively infected mouse embryo cultures was measured by reassociation kinetics with iodinated viral DNA. The data were analyzed by a computer program and indicated the following: before DNA replication approximately 25% of the genome was transcribed into asymmetric transcripts, of which slightly fewer than half of the sequences were recovered from the cytoplasm. After viral DNA replication, approximately 38% of the genome was transcribed (5% as symmetric transcripts), and again less than half of the sequences appeared in the cytoplasm. Both early and late RNA comprised two abundance classes differing about 8- to 10-fold in concentration. Early RNA was a subset of late RNA. The RNA sequences synthesized in late-infected cells in the presence of cytosine arabinoside or cycloheximide were similar to early RNA. Thus, murine cytomegalovirus displays temporal, quantitative, and post-transcriptional controls over gene expression, but the pattern differs considerably from herpes simplex virus.

Herpesviruses contain genomes of the order of 10^8 in molecular weight and would therefore be expected to incorporate a number of control features in their transcription. This has indeed been shown for herpes simplex virus (HSV), which exerts both temporal and quantitative (abundance) controls (2, 18), although HSV types 1 and 2 differ in detail (3). Post-transcription controls have also been demonstrated (8, 18).

The murine cytomegalovirus (MCMV) possesses a genome some 30% larger than other herpesviruses analyzed (13), yet its replication is dependent upon the S-phase of cultivated mouse fibroblasts (15). Because MCMV displays several properties which are not common to other herpesviruses (5, 6), it was of interest to us to examine transcriptional and post-transcriptional controls in MCMV-infected cultures. Furthermore, such a study would provide a base from which we could compare viral gene expression in productive and nonproductive infections. The latter situations have been documented elsewhere (11; M. T. Muller, J. K. Chantler, V. Misra, and J. B. Hudson, submitted for publication). The present communication describes our studies on productively infected mouse embryo cultures, in which the DNA-RNA hybridization data were analyzed by a computer program similar to the one developed by Frenkel and Roizman for analysis of HSV transcription (2).

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MATERIALS AND METHODS

Solutions. Acetate buffer consisted of 0.025 M sodium acetate and acetic acid to a final pH of 5.0. TNE (pH 7.5) consisted of 0.15 M sodium chloride, 0.01 M Tris-chloride, and 0.001 M EDTA; 1× SSC consisted of 0.15 M sodium chloride plus 0.015 M sodium citrate. RNA buffer (pH 5.2) contained 0.1 M sodium chloride, 0.01 M sodium acetate, and 0.001 M magnesium chloride. S1 buffer (pH 4.5) contained 0.3 M sodium chloride, 0.03 M sodium acetate, and 0.001 M zinc sulfate.

Cells and virus. The procedures for the cultivation of mouse embryo and 3T3 cells, propagation of MCMV, and the method of infection have been described in detail elsewhere (13, 15).

Purification of DNA. Virus, purified by differential centrifugation (13), was suspended in TNE buffer. Solid NaCl was added to increase the Na^+ concentration to 1 M. Sodium dodecyl sulfate and predigested Pronase were added to 1% and 1 mg/ml, respectively, followed by incubation at 37°C for 30 min. An equal volume of water-saturated phenol was added, and the mixture was rolled at 65°C for 2 min. The phenol phase was then removed, and the DNA solution was rolled in chloroform until the aqueous phase cleared. Centrifugation at 3,000 rpm for 10 min in an IEC-CS centrifuge was sometimes necessary to clear the aqueous phase. DNA was spooled out in cold 95% ethanol, dissolved in TNE, and precipitated with 95% ethanol. This DNA was purified further by banding in CsCl gradients and recovering the DNA with a density of 1.718 g/ml.

Iodination of viral DNA. Viral DNA was iodi-

nated by the method of Commerford (1), modified to minimize damage or alteration to labeled DNA.

Purified and denatured MCMV DNA, in 5 μl of distilled water, was heated to 65°C for 20 min with 10 μl of acetate buffer, 5 μl of 3×10^{-3} M thallic chloride solution (in acetate buffer), and 2.5 μl of $Na^{125}I$, contained in a stoppered plastic vial.

After diluting with 200 μl of 0.05 M phosphate buffer (pH 6.8), the iodination mixture was applied to a hydroxyapatite column (0.5 by 1 cm). The column was then washed extensively with 0.05 M phosphate buffer until fewer than 40,000 cpm of unreacted ^{125}I were eluted per ml of buffer. Iodinated DNA was then eluted in 0.40 M phosphate buffer.

Fractions containing DNA, in 0.40 M phosphate buffer, were reheated at 65°C for 1 h to convert the intermediate 5-iodo-6-hydroxydihydrocytosine to 5-iodocytosine (1).

Iodinated DNA was dialyzed extensively against 2× SSC and stored at −20°C in 50% glycerol. Only DNA preparations with specific radioactivities of 1×10^7 to 2×10^7 dpm/μg were used, since higher-specific-radioactivity DNAs did not reassociate with acceptable kinetics. The DNAs used reassociated to at least 80% with homologous DNA under our standard hybridization conditions. Viral DNA labeled with [^{3}H]thymidine (13) gave similar results in the transcription analysis, although these were not analyzed in detail. We believe that we were able to obtain satisfactory probes because (i) we sacrificed very high specific radioactivity, (ii) we used pH 5.0 instead of 4.5 during iodination, and (iii) we removed $TlCl_3$ by hydroxyapatite chromatography before the second heating step (17).

RNA purification. Cells were lysed in 10 ml of RNA buffer containing 1% sodium dodecyl sulfate. Proteinase K was added to 100 μg/ml, and the lysate was heated at 65°C for 1 h. The RNA was then extracted and purified by the phenol-chloroform technique (14).

All glassware and solutions used in RNA purification were treated with diethylpyrocarbonate (50 μl/100 ml of water or solution) and then heated to degrade diethylpyrocarbonate.

Nucleic acid hybridization. (i) DNA-DNA. Radioactive viral DNA was mixed with an appropriate amount of the unlabeled DNA to be analyzed in 2× SSC. Calf thymus DNA was added to a total DNA concentration of 500 μg/ml. The mixture was sonically treated for 3 min with the microprobe of a Biosonik sonic oscillator. This procedure sheared DNA into fragments that cosedimented in a 5 to 20% sucrose gradient with ^{125}I-labeled *Drosophila* tRNA (obtained from G. M. Tener). The DNA was then sealed into 100-μl Corning micropipettes, denatured in a boiling-water bath for 20 min, and transferred to a water bath equilibrated at 67°C. Before denaturation, immediately after denaturation, and at various times during the incubation process, sealed tubes were removed and frozen by immersion into ethanol at −20°C, and the contents were added to 1 ml of cold S1 buffer. Single-stranded DNA was degraded by incubating at 37°C with 60 U of S1 nuclease for 1.5 h, and double-stranded DNA was precipitated with cold 10% trichloroacetic acid. Trichloroacetic acid precipitates were trapped on glass fiber filters, washed with ethanol, and counted in a Beckman ISOCap 300 liquid scintillation counter. Under the conditions used, S1 nuclease degraded double-stranded DNA less than 5% and single-stranded DNA at least 98%.

After subtraction of background, S1-resistant counts per minute for each time point were expressed as a percentage of S1-resistant counts per minute in the sample before denaturation.

(ii) DNA-RNA. A 5-ng amount of iodinated MCMV DNA was mixed with 500 to 1,000 μg of the RNA to be tested in 1.0 ml of 2× SSC. After the nucleic acids were sheared by sonic treatment, the hybridization mixture was sealed into 50- or 100-μl micropipettes. Conditions of hybridization and treatment of samples were similar to those outlined for DNA-DNA hybridization in solution, except that S1 buffer contained, in addition, 10 μg of denatured calf thymus DNA per ml (19).

Experimental design and analytical treatment of data. The techniques used in this study were similar to those described by Frenkel and Roizman (2) and involved an analysis of the kinetics of hybridization of trace amounts of radioactive viral DNA with a large excess of unlabeled RNA. Formation of DNA-RNA hybrids was monitored by resistance to the single-stranded nuclease S1.

The basic equation used to describe the kinetics of hybridization was (2):

$$\frac{C}{C_0} = \alpha_1 \cdot e^{-\beta_1 t} + \ldots . \alpha_n \cdot e^{-\beta_n t} + 1 - (\alpha_1 + \ldots . \alpha_n)$$

where $\beta_1 \ldots . \beta_n$ are the products of the molar concentrations of the RNA species ($R_1 \ldots . R_n$) and the rate constant, K. Comparison of the values of β for the different RNA species is then an indication of their relative concentrations in the infected cells. The ratio C/C_0 for each time point t was determined experimentally. Values of α and β were calculated from a plot of C/C_0 versus t fitted to the equation on an IBM 370, model 168 computer, by nonlinear, least-squares optimization of the parameters α_1 through α_n and β_1 through β_n for $n = 1, 2$, etc. The actual curves were generated with the aid of a Hewlett Packard 9810A calculator fitted with a 9862A plotter.

Curves that best fit the equation were obtained by using a nonlinear optimization routine called FLETCH (University of British Columbia nonlinear regression program manual, 1975). FLETCH minimizes the function value, F, or the sum of the squares, defined as:

$$F = \sum_{i=1}^{\text{data points}} \left\{ \frac{C_i}{C_0} - [\alpha_1 \cdot e^{-\beta_1 t_i} + \ldots \alpha_n \cdot e^{-\beta_n t_i} + 1 - (\alpha_1 + \ldots \alpha_n)] \right\}^2$$

where C_i/C_0 is the experimentally determined dependent variable of the fraction of DNA remaining single stranded at time t. It follows, therefore, that the closer the final function value is to 0, the better the fit.

Self-annealing viral DNA (which was always less than 5%) was monitored by incubating the iodinated probe with heterologous RNA under conditions iden-

tical to those utilized for test RNA. A plot of $1 - C_0/C$ versus t for the DNA probe in the presence of heterologous RNA gave a straight line with slope = 1.3×10^{-4} and intercept at 0. For all hybridization reactions, each C/C_0 value was then corrected for self-annealing of the probe by adding to it the corresponding $1 - C/C_0$ value, calculated from the equation: $1 - C/C_0 = 0 + 1.3 \times 10^{-4} \cdot t$.

RESULTS

Temporal and quantitative control of MCMV transcription. Viral DNA synthesis in MCMV-infected mouse fibroblasts commences at 8 h postinfection (p.i.); therefore, RNA was extracted from cultures at 6 h p.i. for "early RNA" and at 24 h p.i. for "late RNA."

These two RNA populations were hybridized to ^{125}I-labeled MCMV DNA, and the results were analyzed by the computer program described above. The analysis revealed that both early and late viral RNA comprised two abundance classes (Fig. 1). Further details are summarized in Table 1 and indicate that: (i) at 6 h p.i. cells contained transcripts from approximately 23% (17.7 + 5.3) of the viral genome or, assuming asymmetric transcription (see below), 46% of its coding capacity; (ii) at 24 h p.i. 37% (18.7 + 18.7) of the viral DNA was represented in RNA transcripts, i.e., 74% of the coding capacity; (iii) both early and late RNA contained two classes of transcripts differing approximately 8- to 10-fold in concentration. Similar data were obtained for RNA extracted from MCMV-infected 3T3 cells. In addition, similar data were obtained with ^{3}H-labeled probe DNA, although these data were not analyzed by the computer program.

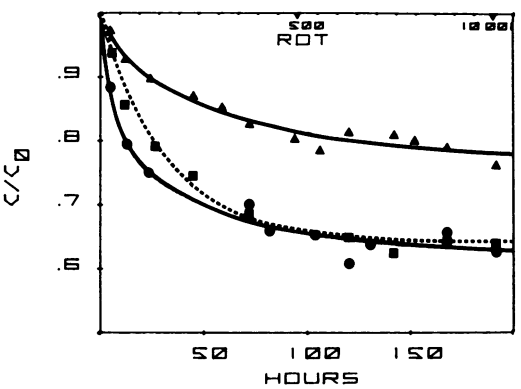

FIG. 1. *Transcriptional analysis of early and late RNA. 500 μg of early RNA per ml (▲), 500 μg of late RNA per ml (●), and a mixture of 250 μg each of early and late RNA per ml (■) were analyzed in hybridization reactions with iodinated MCMV DNA.*

In five different experiments, the sum of the $\alpha_1 + \alpha_2$ values varied over the range of 23 to 26% of the viral genome for early RNA and 36 to 39% for late RNA, the ratio of α_1 to α_2 remaining approximately constant. The ratio of β_1 to β_2 ranged from 7.2 to 10.0 for early RNA and 8.7 to 13.7 for late RNA.

Comparison of early and late RNA sequences. To determine whether early RNA was a subset of late RNA, equal amounts of the two populations were mixed and hybridized to MCMV DNA, in comparison with each population hybridized separately. If the two populations contained different sequences, the curves should be additive. This was not observed, however (Fig. 1), and we conclude that all or most of the early RNA sequences were also present in late RNA.

Transcription of MCMV in the presence of inhibitors. Viral RNA synthesis was analyzed in infected cells in the presence of cytosine arabinoside (ara-C) or cycloheximide. The characteristic early cytopathic effect caused by MCMV, i.e., cell swelling, which occurs around 6 to 8 h p.i., was not affected by the presence of ara-C, although no infectious virus was produced. Viral DNA replication was inhibited, however, as shown by the lack of increase of viral genomes in ara-C-treated cells (Fig. 2). Analysis of viral transcription in these cells revealed that the RNA comprised two abundance classes, coded by 5 and 17% of the viral genome,

TABLE 1. *Best-fit α and β values for RNA extracted from MCMV-infected cells*[a]

Sample	Best fit (n = 1 or 2)	α_1	α_2	$\alpha_1 + \alpha_2$	β_1	β_2	β_1/β_2
(E) 6-h RNA	2	0.054	0.18	0.23	0.105	0.014	7.5
(L) 24-h RNA	2	0.18	0.18	0.37	0.16	0.018	8.9
6-h RNA + 24-h RNA (summation of E + L)	1	0.36		0.36	0.03		
RNA cycloheximide-treated cells	2	0.078	0.19	0.27	0.152	0.010	15.0
RNA cycloheximide-treated cells + 6-h RNA (E)	2	0.052	0.19	0.24	0.105	0.018	5.8
Ara-C RNA	2	0.053	0.18	0.23	0.166	0.026	6.4

[a] E, Early; L, late; n, number of classes of RNA differing in concentration (abundance); α_1 and α_2, fraction of genome saturated by complementary RNA sequences; β_1 and β_2, relative concentrations of classes of RNA.

in a ratio of 6 to 1, similar to early RNA (Fig. 3 and Table 1).

Cycloheximide blocks herpesvirus DNA synthesis indirectly by inhibiting early protein synthesis (3, 20). The RNA extracted from cycloheximide-treated MCMV-infected cells was subjected to hybridization analysis, as above. The results (Fig. 4 and Table 1) indicate that this RNA is also similar qualitatively (summation hybridization test) and quantitatively (abundance classes) to early RNA.

Symmetrical versus asymmetrical transcription. To investigate the possibility of symmetrical transcription, which was found to occur to a significant extent in HSV type 1-infected cells (7, 9), RNA samples were self-annealed before their standard hybridization with MCMV DNA. Under such conditions symmetrical transcripts would form double-stranded RNAs and would, therefore, be unavailable for subsequent interaction with the ^{125}I-labeled viral DNA. A depression in the saturation hybridization value would result, the amount of this depression representing the portion of the genome transcribed symmetrically. The results are shown in Fig. 5 and indicate that for both early and late RNA the extent of symmetrical transcription was below the level of detection.

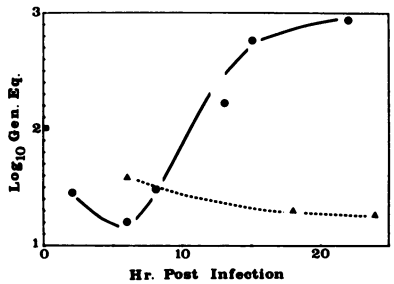

FIG. 2. *Enumeration of MCMV genomes in infected mouse cells in the presence and absence of ara-C. Mouse embryo monolayers in 60-mm Falcon tissue culture dishes were infected with MCMV at a multiplicity of approximately 10 PFU/cell. After infection, half the dishes were overlaid with minimal essential medium plus 10% serum containing 50 μg of ara-C per ml. The remainder were overlaid with minimal essential medium plus 10% serum without ara-C. At various times after infection, cells were harvested and analyzed for their content of viral genomes, as described previously (12). Symbols: ●, untreated cells; ▲, ara-C-treated cells.*

Nuclear and cytoplasmic RNA. RNA was extracted separately from nuclear and cytoplasmic fractions of cells harvested at 6 and 24 h p.i. and subjected to hybridization analysis (Fig. 6). In the early RNA, approximately 23% of the viral genome was represented as transcripts in the nucleus, but only 11% was represented in the cytoplasm. The corresponding values for late RNA were 37% in the nucleus and 15% in the cytoplasm.

The RNA derived from early nuclear and cytoplasmic fractions and from late cytoplasm consisted solely of asymmetrical transcripts (Fig. 6). In contrast, late nuclear RNA contained symmetrical transcripts from approximately 5% of the viral genome. Thus, symmetrical transcription represents a small number of viral genes and contributes relatively little to total RNA concentration (it was not detected in unfractionated whole-cell RNA [Fig. 5]), and the RNAs concerned are not subsequently transported to the cytoplasm.

DISCUSSION

Transcription of the MCMV genome in productively infected cells was analyzed by techniques similar to those described by Frenkel and Roizman (2). This analysis permitted us to determine that MCMV displays temporal and

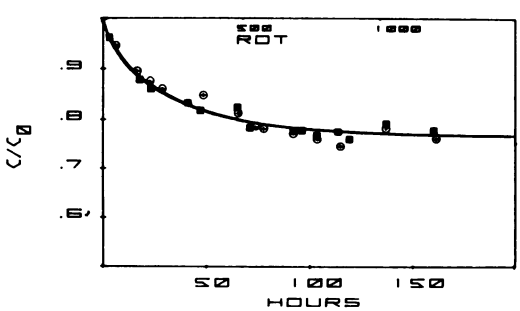

FIG. 3. *Transcriptional analysis of early RNA and RNA extracted from ara-C-treated cells. 700 μg of early (6 h) RNA per ml (■) and 700 μg of RNA extracted from ara-C-treated cells per ml (⊕) at 24 h p.i. were analyzed in hybridization reactions with iodinated MCMV DNA.*

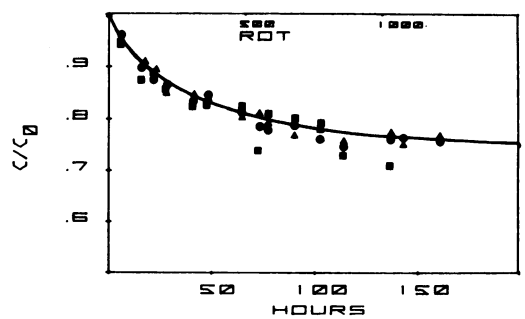

FIG. 4. *Transcriptional analysis of early RNA, RNA from cells treated with cycloheximide, and a mixture of the two RNAs. Symbols: ●, 700 μg of early (6 h) RNA per ml; ■, 700 μg of RNA from cycloheximide-treated cells (6 h p.i.) per ml; ▲, 350 μg of early RNA per ml + 350 μg of cycloheximide RNA per ml.*

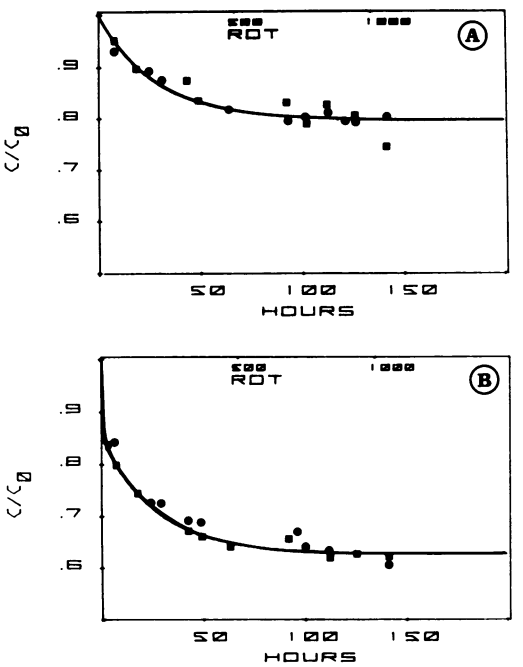

FIG. 5. *Analysis of early and late RNA for symmetric transcripts. Early RNA (A) and late RNA (B) were preannealed in 2× SSC at 67°C to an R_0t of 2,500 (10 mg/ml for 24 h) before mixing with denatured ^{125}I-labeled MCMV DNA and sealing in tubes. Before hybridization at 67°C, half the tubes in each batch were heated in boiling water to denature any double-stranded RNA formed. The remaining tubes were directly incubated at 67°C. Symbols: ●, preincubated RNA; ■, preincubated, denatured RNA.*

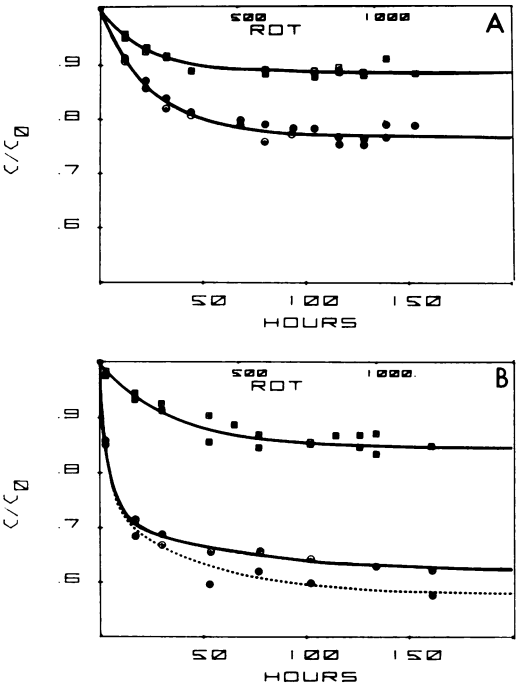

FIG. 6. *Transcriptional analysis of cytoplasmic and nuclear RNA. Conditions of infection were similar to previous experiment. Cells were fractionated by Nonidet P-40 treatment (9), and the RNA was extracted separately from the washed nuclei and cytoplasm. (A) Early cytoplasmic RNA (6 h p.i.): ⊞, preincubated; ■, preincubated and then denatured. Early nuclear RNA (6 h p.i.): ⊕, preincubated; ●, preincubated and then denatured. (B) Late cytoplasmic RNA (24 h p.i.): ⊞, preincubated; ■, preincubated and then denatured. Late nuclear RNA (24 h p.i.): ⊕, preincubated; ●, preincubated and then denatured.*

quantitative controls over transcription and further post-transcriptional controls. Similar levels of control were also found to operate in HSV-infected cells (2, 3, 7, 8, 16, 18, 21) and in Epstein-Barr virus-infected cells (4), although HSV types 1 and 2 differ in detail (3).

In cells productively infected with MCMV, approximately 50 and 75% of the coding capacity of the genome is utilized, respectively, during the early and late phases of the virus growth cycle. Early transcripts were either completely retained throughout the growth cycle or were continuously synthesized. Both early and late RNA populations comprised two classes differing about 8- to 10-fold in concentration. The scarce class represented approximately 18% of the genome at early and late times, whereas the abundant class increased from 5% of the genome at early times to 18% at late times. Although early RNA was found to be a complete subset of late RNA, we cannot yet conclude from these data that the "switch" from early to late transcription simply represents an amplification of the scarce early genes, because we cannot identify individual gene transcripts. Also, we do not know to what extent the extracted RNA populations reflect the dynamics of synthesis and degradation. The analysis presented here merely informs us about viral RNAs existing in the cells at the times of harvest. A previous study of pulse-labeled viral RNA did, however, reveal at least two distinct phases of viral RNA synthesis, corresponding to our early and late designations (14). The data suggest that symmetric transcription of MCMV is not a significant feature, in contrast to HSV (7, 9), although we realize the difficulty of making precise measurement of double-stranded RNAs under our experimental conditions.

It is interesting that 10 to 15% of the MCMV genome was apparently not transcribed at all, or, if it was, then the transcripts were present in very small numbers or were rapidly degraded. A

similar unresolved question concerns the fate of those nuclear transcripts not transported to the cytoplasm (about 50% of early transcripts and 60% of late transcripts). Although eucaryotic transcripts generally suffer considerable loss of their sequences before emerging as messengers into the cytoplasm, it has been assumed that the lost sequences represent "noncoding" regions of the DNA (10). It is conceivable that a large viral genome such as MCMV may also contain noncoding regions. However, we cannot rule out the possibility that the viral genes unaccounted for might simply be genes that are only transcribed or processed to yield one or a few copies of mRNA(s) and which might be beyond the sensitivity of the technique used. It is also possible that the less-than-100% transcription observed could be due to a technical limitation, i.e., the difficulty of obtaining 100% reassociation of RNA with the probe DNA, although the observation was made also with [^{3}H]thymidine-labeled viral DNA. Nevertheless, it is clear that the herpesviruses display considerable individuality with regard to transcriptional control, as well as to other properties examined, making it difficult to classify them into discrete subgroups.

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