Control of transcription of the reovirus genome

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ABSTRACT

The ten double-stranded RNA segments of the reovirus genome are not transcribed at equal frequencies until later times during the course of infection. When an inhibitor of protein synthesis such as cycloheximide was added to infected cells at the beginning of infection, only four of the ten segments were transcribed. By two hr post infection, five and possibly seven of the segments were being transcribed. By four hr post infection all ten segments were being transcribed but not yet at equal relative frequencies. The transcription pattern at intermediate (4 hr) and late (10 hr) times were verified without using cycloheximide. A repressor present in the host cell may be responsible for controlling transcription of the reovirus genome.

INTRODUCTION

The genome of reovirus consists of double-stranded RNA (dsRNA) with a molecular weight of approximately 16 x 10^6 daltons[1,2,3]. When the dsRNA is extracted from purified virions and analyzed by electrophoresis on polyacrylamide gels, ten bands are observed which correspond to single segments of the viral genome[2,3].

Recent evidence indicates that the viral genome exists *in situ* as a segmented structure[4,5]; that is, both strands of the dsRNA duplex are covalently discontinuous at regions corresponding to the ends of the isolated segments. How the genome segments are held in their correct alignment is not yet known but it has been known for some time that in the infected cell, the viral genome is transcribed as if there were discontinuities

in the structure[6,7]. Thus, when messenger RNA (mRNA) isolated from infected cells late in the replicative cycle is hybridized with denatured genomic dsRNA and analyzed by polyacrylamide gel electrophoresis ten mRNA hybrids can be detected[3]. Furthermore the ten mRNA hybrids and the ten reassociated segments migrate at the same rate and from this observation it can be calculated that at least 99 percent of the total genome is transcribed late in infection[8].

When an inhibitor of protein synthesis such as, cycloheximide is added at the time of infection only four of the ten segments are transcribed[1,3], presumably by the virion-associated RNA-dependent RNA polymerase[9,10] (transcriptase). There is the obvious question whether this early mRNA pattern represents the true state of affairs or whether the results are influenced by some unknown side effect of cycloheximide.

In this report we show that at early times during the course of infection the dsRNA segments of the reovirus genome are turned on in a sequential manner while at late times all segments are transcribed with equal frequency.

MATERIALS AND METHODS

Cells and Virus. Mouse fibroblasts (L-cells) were grown in suspension culture in spinner modified minimal essential medium (Grand Island Biological Company) supplemented with 5% fetal calf serum. The Dearing strain of reovirus type 3 was used. Cells were infected at a concentration of 10^7 cells/ml using a multiplicity of 20 plaque forming units per cell. Adsorption was carried out for 1 hr at 4C to maximize synchrony of infection[1]. Infected cells were then centrifuged and resuspended at

0.5×10^6 cells/ml in medium pre-warmed to 37C and containing 0.5 μg/ml actinomycin D and 2% fetal calf serum. Time of resuspension at 37C was taken as zero time post infection (p.i.). Infected cultures were incubated routinely at 37C.

Chemicals. [5-^{3}H]uridine (25Ci/mmole) and [2-^{14}C]uridine (50mCi/mmole) were obtained from Schwartz Mann. [^{3}H]Sodium borohydride (30Ci/mmole) and ^{32}P orthophosphate was from New England Nuclear. Ribonuclease-free DNase was purchased from Worthington. Cycloheximide (Actidione) was obtained from Upjohn Company, Michigan.

Preparation of RNA and Hybridization Procedures. Labelled dsRNA was prepared from purified virus particles as described previously[12]. Conditions for labelling single-stranded RNA (ssRNA) in vivo with ^{3}H uridine were the same as described previously[1] except that high concentrations of Actinomycin D (2 μg/ml) were not added prior to labelling and cycloheximide (20 μg/ml) was not added except where indicated. RNA was extracted from infected cells by the phenol-SDS method[1] and the ssRNA effectively separated from dsRNA by two precipitations from 1 M NaCl at 0C. Recovery of RNA following a previously described hybridization procedure[1] was 70 to 80 percent.

Polyacrylamide Gel Electrophoresis. After annealing purified ^{3}H-labelled ssRNA with excess, denatured ^{14}C-labelled dsRNA the hybrids were precipitated by ethanol and recovered by centrifugation. The precipitate was dissolved in 0.5 ml of 10 mM Tris (Cl), pH 7.2, 1 mM EDTA, heated at 72C for 7 min then quickly chilled in ice. The solution was adjusted to 1 M NaCl and kept at 0C overnight to precipitate ssRNA and partial dsRNA

structures. Following centrifugation, the hybrids were recovered from the supernate by precipitation with ethanol then analyzed by electrophoresis in 5 percent polyacrylamide gels[3]. The gels were stained with methylene blue, frozen and sliced with a microtome to a thickness of 0.35 mm[3]. Gel slices were solubilized in 30 percent H_2O_2 and the radioactivity determined by liquid scintillation counting.

RESULTS

Determination of Relative Transcription Frequencies of the Genome Segments. Synthesis of reovirus progeny dsRNA in infected L-cells begins between 4 to 5 hr p.i. and is proceeding

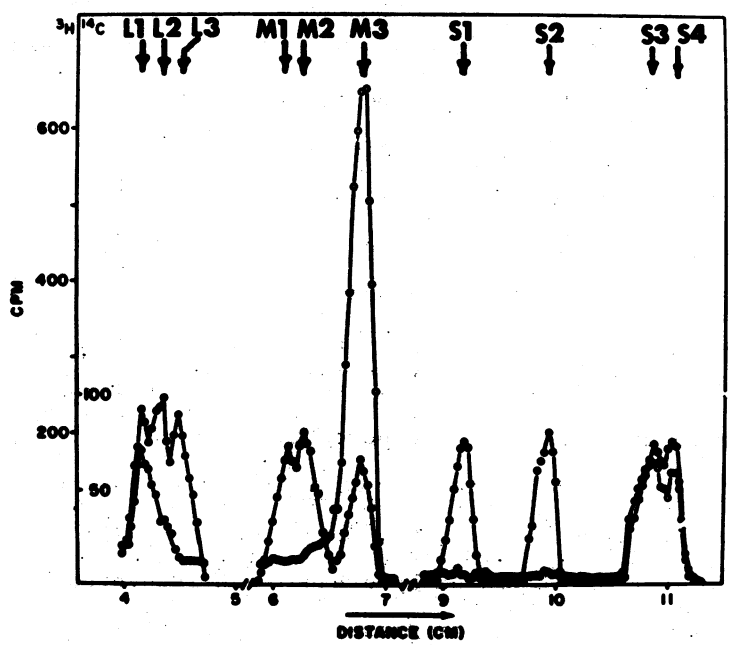

Figure 1. Polyacrylamide gel electrophoretic analysis of the hybrids formed between viral dsRNA and mRNA synthesized in infected cells when cycloheximide was present from the beginning of infection. Closed circles, ^{14}C-labelled genome hybrids. Open circles, ^{3}H-labelled mRNA hybridized with genome dsRNA.

at its maximum rate about 7 to 8 hr p.i. If one adds an inhibitor of protein synthesis such as, cycloheximide during this time synthesis of dsRNA ceases while synthesis of ssRNA continues[16,17]. Cycloheximide added at zero time p.i. blocks the synthesis of protein and progeny dsRNA but does not prevent uncoating of the virus in the cell[18], nor does it completely inhibit transcription of the viral genome[1,3]. This is shown clearly in Figure 1 where four of the ten segments, namely segments L-1, M-3, S-3, and S-4 were transcribed when cycloheximide was present from the beginning of infection. In this and subsequent experiments the mRNA, labelled with ^{3}H uridine was hybridized with a large excess of ^{14}C-labelled dsRNA obtained from purified virus. Under the conditions used, the efficiency of hybridization decreases with increasing length of the dsRNA segments and also from one experiment to another. However, since the dsRNA is in large excess the ^{14}C label provides an internal standard by means of which the hybridization results can be quantitated. Thus the $^{3}H/^{14}C$ ratio of each peak provides a measure of the relative frequency of transcription for each segment within a given gel. However, if the conditions for labelling the mRNA are not precisely the same, the $^{3}H/^{14}C$ ratios obtained from one experiment to another cannot be compared. For this reason we have normalized the results by setting the $^{3}H/^{14}C$ ratio of segment M3 equal to 10 for each experiment.

Transcription of the Viral Genome in the Presence of Cycloheximide. Such an analysis was performed on the data shown in Figure 1 and the results are presented in Table 1. The largest segment (segment L-1) and the two smallest segments (segments S-3 and S-4 were transcribed with equal frequencies. The only other

TABLE 1
Ratios of $^{3}H/^{14}C$ in Hybrids Formed Between Viral dsRNA and Virus-induced mRNA Synthesized in the Presence of Cycloheximide[1]

Component[2]	Labelling Period With Cycloheximide (HR)			
	0-8	2-10	4-12	10-13
L-1	2.1	3.4	6.1	10.7
L-2	-	±	4.4	9.6
L-3	-	-	3.8	9.6
M-1	-	-	3.6	9.8
M-2	-	2.6	4.4	11.1
M-3	10	10	10	10
S-1	-	-	7.5	11.1
S-2	-	±	6.8	10.0
S-3	2.2	6.1	14.2	12.6
S-4	2.2	5.6	14.7	11.1

1. Ratios within each column normalized to a value of 10 for segment VI.
2. Refers to the ten genome components separated by polyacrylamide electrophoresis.

segment transcribed under these conditions was segment M-3 and it is still far from clear why this segment was transcribed at a frequency 5 times that of the other three early segments. If we allow infection to proceed normally for 2, 4 or 10 hr p.i. prior to adding cycloheximide, then analyze the transcription products synthesized following addition of the drug, there is an apparent transition from the early pattern (zero time p.i.) to the late pattern (10 hr p.i.) (Table 1). Analysis of the transcription pattern following addition of cycloheximide at 2 hr p.i. shows that in addition to the four early segments, segment M-2 and to a lesser extent, segments L-2 and S-2 are now being transcribed

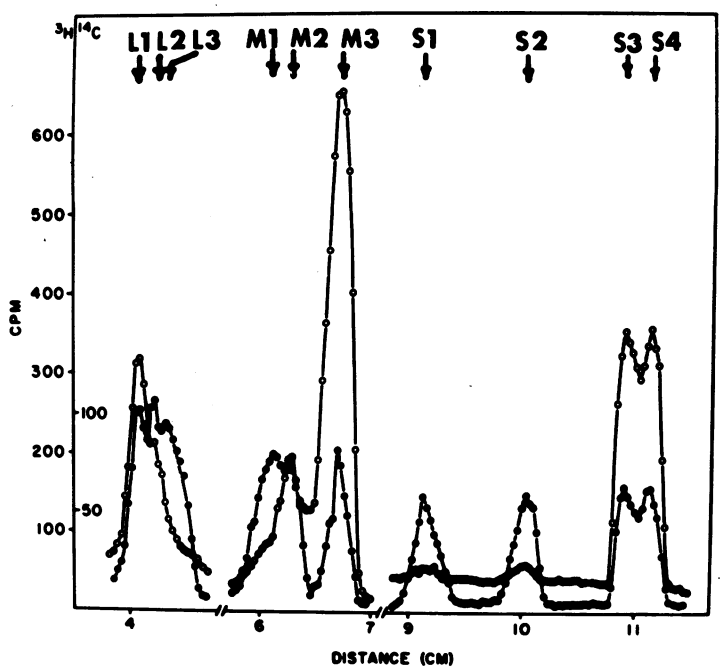

Figure 2. Polyacrylamide gel electrophoretic analysis of the hybrids formed between viral dsRNA and mRNA synthesized in infected cells following addition of cycloheximide at 2 hr p.i. Closed circles, ^{14}C-labelled genome hybrids. Open circles, ^{3}H-labelled mRNA hybridized with genome dsRNA.

(Fig. 2 and Table 1). By 4 hr p.i. all segments are being transcribed but not yet at equal frequencies whereas by 10 hr p.i. all segments are being transcribed at approximately equal frequencies (Table 1). These data suggest that in the absence of protein synthesis from zero time only four of the ten segments of the reovirus genome can be transcribed. Furthermore, the remaining six late segments seem to be turned on in a sequential fashion. It would appear from the data presented so far that cycloheximide freezes the pattern of transcription that exists at the time of its addition thus preventing any further derepression of transcription of the various segments.

Transcription of the Viral Genome in the Absence of Cycloheximide. In the above experiments labelling of the ssRNA was preceeded by the addition of cycloheximide and it could be argued that these results are due to some artifactual side effect of the drug. To obviate this argument the transcription pattern 4 to 5 hr p.i. was examined without using cycloheximide. The pattern obtained is shown in Figure 3 and the $^{3}H/^{14}C$ ratios,

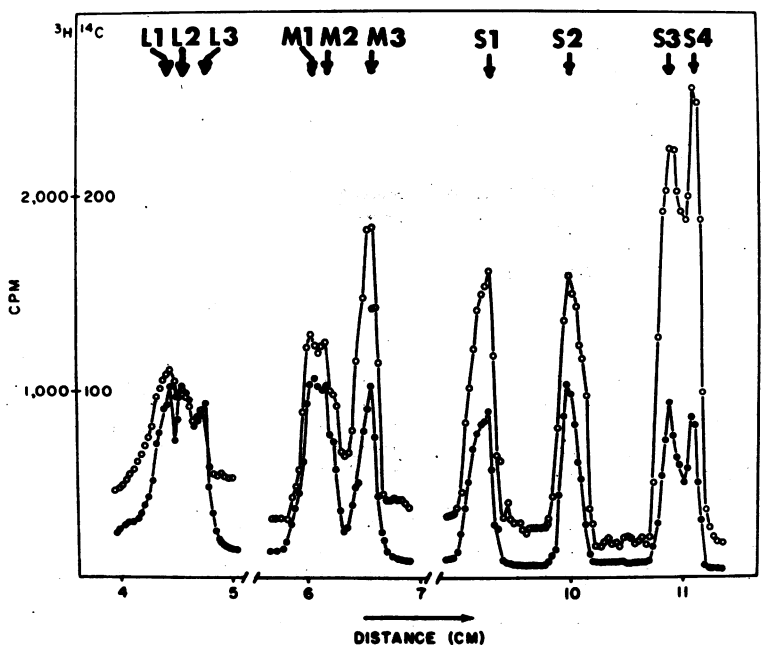

Figure 3. Polyacrylamide gel electrophoretic analysis of the hybrids formed between viral dsRNA and viral-specific mRNA synthesized between 4 and 5 hr p.i. in the absence of cycloheximide. Closed circles, ^{14}C-labelled genome hybrids. Open circles, ^{3}H-labelled mRNA hybridized with genome dsRNA.

normalized to segment M-3 are presented in Table 2. It is clear that the relative frequencies of transcription of the genome segments between 4 to 5 hr p.i. in the absence of cycloheximide closely approximates those frequencies obtained over the 8 hr

TABLE 2

Ratios of $^{3}H/^{14}C$ in Hybrids Formed Between Viral dsRNA and Virus-induced mRNA Synthesized in the Absence of Cycloheximide[1]

Component[2]	Labelling Period Without Cycloheximide (HR)	
	4-5	10-11
L-1	4.2	11
L-2	3.0	10
L-3	2.1	10
M-1	4.2	14
M-2	3.7	11
M-3	10	10
S-1	6.9	11
S-2	8.4	12
S-3	13	14
S-4	15	12

1. Ratios within each column normalized to a value of 10 for segment VI.
2. Refers to the ten genome components separated by polyacrylamide gel electrophoresis.

period following addition of cycloheximide at 4 hr p.i. (Table 1). This result supports the contention that cycloheximide has the effect of "locking in" the system to the transcription pattern prevailing at the time of its addition. Transcription occurring late in infection (10 hr p.i.) was also examined in the absence of cycloheximide (Fig. 4). The $^{3}H/^{14}C$ ratios for each peak is presented in Table 2, and there is no substantial difference in the ratio from one peak to another. Similar results were obtained whether the labelling period was 30 min or 2 hr which means that

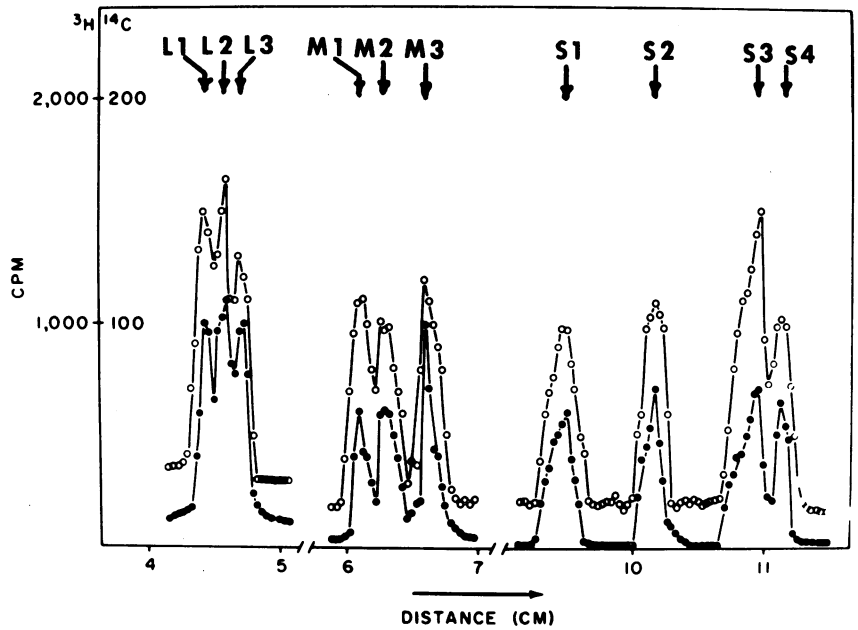

Figure 4. Polyacrylamide gel electrophoretic analysis of the hybrids formed between viral dsRNA and mRNA synthesized in infected cells between 10 and 11 hr p.i. in the absence of cycloheximide. Closed circles, ^{14}C-labelled genome hybrids. Open circles, ^{3}H-labelled mRNA hybridized with genome dsRNA.

late in the infectious cycle all ten segments of the genome are transcribed at approximately the same relative frequency.

DISCUSSION

Inhibitors of protein synthesis, particularly chloramphenicol, have long been used to demonstrate early transcription of phage genomes. We have used cycloheximide for a similar purpose in the reovirus system. As reported previously[3] and illustrated in Figure 1, when cycloheximide is present from the beginning of infection only four of the ten segments are transcribed (Table 1), and these segments by definition, are associated with early viral functions. These early viral transcripts must be

synthesized from the parental infecting virus by the virion associated transcriptase. Thus, in principle, during the course of viral infection it should be possible to see a transition from the early transcription pattern of Figure 1 to the latter pattern of Figure 4. However, in practice relatively little mRNA is synthesized for at least 3 hr p.i. Consequently it is difficult to show whether a sequential turning-on of the latter segments occurs without using cycloheximide. This drug has the effect of freezing the pattern of transcription thus permitting the accumulation of detectable amounts of mRNA. The results of four such experiments summarized in Table 1, clearly show a sequential turning on of the genome segments at early times during infection.

This sequential turning on of the genome segments is inconsistent with a previous report which showed that the transcription pattern remained constant over two hour periods from 2 to 8 hr p.i.[18]. However, these investigators did not use cycloheximide and furthermore, since radioautography was used to measure the mRNA hybrids in polyacrylamide gels it was not possible to correct for the differences in efficiency of annealing between the genomic segments. We also examined the transcription patterns from 4 to 5 hr and from 10 to 11 hr p.i. without using cycloheximide. Between 4 and 5 hr p.i. all the segments are being transcribed (Fig. 3) but not yet at the same relative frequencies (Table 2). We conclude that Figure 3 represents a transition between early and late transcription, and since the result was obtained without using cycloheximide it strengthens the argument that early viral functions are associated with genome segments L-1, M-3, S-3 and S-4. Perhaps the most compelling evidence in support of the early mRNA concept

is the fact that when cells are infected with reovirus in the presence of cycloheximide and incubated, they accumulate the 4 early mRNA's which can then be translated into 4 early polypeptides upon removal of the drug (Van Alstyne and Graham, unpublished data). This result proves that the viral transcripts made in the presence of cycloheximide are functional mRNA's. It is not until late in the course of infection (e.g., 10 hr or later, Fig. 4) that all ten segments are transcribed with equal frequency (Table 2).

What controls the switch over from early to late transcription *in vivo* is still not understood. No one has yet been able to obtain the early transcription pattern *in vitro* with the viral core RNA polymerase. Parental subviral particles isolated from cells infected in the presence of cycloheximide also synthesize all ten mRNA's in the *in vitro* system[19]. In addition, for seven groups of conditional lethal (temperature sensitive) mutants of reovirus transcription of all ten segments occurs in cells infected at the non-permissive temperature[20,21]. Three of these groups are dsRNA$^-$ and at least one might have been expected to show an early transcription pattern. Perhaps the simplest explanation at the present time invokes a repressor in the host cell which prevents transcription of the late segments. This repressor could be inactivated by an early viral protein thus derepressing transcription of the six late segments. We are presently investigating this possibility.

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