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Regulated Transcription of the Genomes of Defective Virions and Temperature-Sensitive Mutants of Reovirus

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Defective reovirus, which lacks the largest (L_1) of the 10 double-stranded (ds) RNA genomic segments, attaches to L cells and is uncoated in the same way as reovirus. The defective genome does not replicate in the cells, but it is transcribed. During the first 5 h after infection, three of the genomic segments, M_3, S_3, and S_4, are more frequently transcribed than the remaining six segments. During the succeeding 5 h, there is a transition to a situation in which all nine segments are transcribed at the same relative frequencies. Since the class C *ts* mutation has been allocated to the L_1 segment (Spandidos and Graham, 1975) the transcription of the C mutant genome was investigated in cells infected with it at the nonpermissive temperature, at which the parental genome does not replicate. Genomic segments L_1, M_3, S_3, and S_4 are predominantly transcribed at early times, and later all 10 segments are transcribed with the same relative frequencies. Transcription of the defective viral genome and the C mutant genome is therefore regulated in the same way as previously found for wild-type virus (Nonoyama, Millward, and Graham, 1974), and the regulation is independent of genome replication. Apparently the L_1 segment function is involved in dsRNA synthesis but not in regulating the early to late transcription. It is suggested that a cellular repressor may be involved in this regulation and that derepression might be effected by one of the early viral gene products. Virion transcriptase activity was studied in vitro with cores prepared by chymotrypsin digestion of purified defective and standard virions. For both genomes the relative frequencies of transcription of the dsRNA segments are inversely proportional to their molecular weights. These results can be accounted for in a model that postulates each segment to be transcribed independently of the other. The same model with certain restrictions can describe the in vivo transcription of the viral genome.

During the course of infection with reovirus all 10 of the double-stranded (ds) RNA segments of the viral genome are transcribed into single-stranded (ss) RNAs of equivalent lengths. Previous work has shown that this transcription is regulated; very early in the infectious cycle a limited number of the genomic segments is transcribed, and later all of the segments are transcribed with similar frequencies (14, 15, 28). Those segments involved in early transcription were defined by infecting cells in the presence of cycloheximide and identifying the viral ssRNA's then produced. Under these conditions segments L_1, M_3, S_3, and S_4 were the ones predominantly transcribed (14, 15). More recently it has been found that the ssRNA's accumulated in the presence of cycloheximide are functional mRNA's since they are translated into corresponding proteins soon after the removal of the inhibitor (12). Presumably one of the early gene products controls transcription of the remainder of the genome, but the mechanism is far from clear.

One means of studying this regulation should be through the use of temperature-sensitive (*ts*) mutants of reovirus and a number of such mutants are known (4). They have been grouped into seven classes by the genetic recombination frequencies found in pairwise crosses (3, 4). Each class is thought to represent mutations in a different segment of the genome and, since there are 10 segments in this virus, mutations in seven of them are therefore known. Three classes of *ts* mutants (C, D, and E) synthesize no dsRNA (dsRNA$^-$) when cells are infected with them under nonpermissive conditions (2, 9) and thus have the mutations in early functions. Nevertheless, none of these mutants has been implicated in transcriptional control: all 10 segments have been found to be transcribed with the three classes of dsRNA$^-$ mutants at the nonpermissive temperature and the early

transcription pattern has not as yet been observed (2, 9).

Isolation of defective virions completely lacking the L_2 segment of the genome (27) has provided a further opportunity to study transcription in this system. The L_1 segment is one of the four early segments. In the present paper it is shown that when cells are "infected" with these defective virions there is a temporal control over transcription of their genomes similar to that found for infectious virions, even though the defective genome is not replicated. Since we have obtained evidence that the L_1 segment bears the class C *ts* mutation (26), we have reinvestigated the transcription of the C mutant genome at nonpermissive temperature and found that it too displays a transition from an early to late pattern in the synthesis of ssRNA in the absence of dsRNA replication.

MATERIALS AND METHODS

Cells and virus. L cells were grown in suspension culture in Eagle minimum essential medium supplemented with 5% fetal calf serum.

The following strains of reovirus type 3 have been used as previously described: wild-type strains R_1 and R_2 (27), the deletion mutant $R_1d(L_1)$ obtained from the R_1 strain (27), and the class C *ts* mutant $R_2C(447)$ (4). Purified virus was used for all infections and prepared as previously described (27).

Buffers, chemicals, and isotopes. STE buffer (0.01 M) contains 0.01 M NaCl, 0.05 M Tris-chloride (pH 7.4), and 0.001 M EDTA. STE buffer (0.3 M) contains 0.3 M NaCl in place of 0.01 M NaCl. Sodium dodecyl sulfate (SDS) buffer contains 0.1 M NaCl, 0.01 M Tris-chloride (pH 7.4), and 0.5% SDS. Lysis buffer contains 0.01 M Tris-chloride (pH 7.6), 2 mM $MgCl_2$, 0.15 M KCl, and 0.05% Triton X-100. SSC contains 0.15 M NaCl, 0.15 M sodium citrate (pH 7.4). Phosphate-buffered saline (pH 7.3) contains 0.13 M NaCl, 2.7×10^{-3} M KCl, 8.2×10^{-4} M Na_2HPO_4, 1.5×10^{-3} M KH_2PO_4, 9.1×10^{-4} M $CaCl_2$, and 5×10^{-4} M $MgCl_2$. RNase was obtained as the crystalline enzyme from Worthington Biochemicals Corp. [2-^{14}C]uridine (50 mCi/mol) was obtained from New England Nuclear Corp.

Conversion of defective reovirions into subviral particles (SVP) in infected cells (uncoating). ^{32}P-labeled $R_1d(L_1)$ virions were adsorbed to monolayers of L cells for 1 h at 4 C at a multiplicity of infection (MOI) of 1,000 particles/cell. Unadsorbed virions were removed by washing the cells three times with phosphate-buffered saline and then 5 ml of Eagle minimum essential medium containing 2% heat-inactivated fetal calf serum was added and the monlayers were incubated at 37 C. After 3 h the cells were scraped from the dishes, centrifuged, and washed with phosphate-buffered saline, and a cytoplasmic extract was prepared. To do this 2 ml of lysis buffer (0.01 M Tris-hydrochloride, pH 7.6, 2 mM $MgCl_2$, 0.15 M KCl, 0.05% Triton X-100) was added, and the mixture was stirred vigorously with a Vortex mixer and centrifuged at 3,000 $\times$ *g* to remove nuclei and large particulate material. To the supernatant cytoplasmic fraction were added [^{3}H]uridine-labeled R_2 virions to act as a subsequent density marker and sufficient solid CsCl to give an average density of 1.37 g/ml. This mixture was centrifuged for 20 h at 45,000 rpm in an SW65 rotor, and samples were taken after puncturing the bottom of the tube and these were analyzed for trichloroacetic acid-precipitable radioactivity.

Preparation of ssRNA from virus-infected cells. Suspension cultures of L cells were infected at MOIs of 20 PFU/cell for the $R_2C(447)$ mutant or 1,000 particles/cell for the defective $R_1d(L_1)$ virions. Adsorption of virus was carried out for 1 h at 4 C at a cell concentration of 5×10^6/ml and the cells were then centrifuged and resuspended in Eagle minimum essential medium containing 2% heat-inactivated fetal calf serum and 0.5 μg of actinomycin D per ml. The final volume of culture was 100 ml, containing 5×10^5 cells/ml. Cultures containing defective virus were incubated at 37 C; those infected with the C mutant were incubated at the nonpermissive temperature of 39 C. Virus-specific RNA was labeled by the addition of 5 μCi of [^{3}H]uridine per ml added at times to be specified for each experiment. At the end of the period of labeling, the cells were centrifuged and washed with phosphate-buffered saline, and the RNA was extracted with phenol at 60 C in the presence of 0.5% SDS and 0.05 M sodium acetate buffer, pH 5.3. NaCl was added to the extract to give a final concentration of 0.3 M, and the RNA was precipitated by adding 3 volumes of ethanol. The RNA was dissolved in SDS buffer, passed through a column of Sephadex G-25 which had been equilibrated with SDS buffer, and again precipitated by the addition of 3 volumes of ethanol. This procedure removed low-molecular-weight labeled material. If the RNA, which contained viral ssRNA and dsRNA, was to be used for rate zonal sedimentation analysis, it was dissolved in SSC, applied to a 15 to 30% gradient of sucrose containing SSC, and centrifuged at 25,000 rpm for 24 h in an SW27.1 rotor.

Prior to using the viral ssRNA for hybridization analysis, any viral dsRNA had to be removed. To do this the precipitate of RNA obtained after Sephadex G-25 chromatography was dissolved in 0.3 M STE buffer and passed through a column of Sephadex G-100 to remove labeled tRNA. Viral RNA was precipitated from the column effluent by addition of 3 volumes of ethanol. This precipitate was dissolved in 0.3 M STE buffer, NaCl was added to a concentration of 1 M, and the mixture was left for 18 h at 4 C to precipitate the ssRNA. The ssRNA was then dissolved in 0.01 M STE buffer and used in hybridization experiments. The supernatant from the 1 M NaCl precipitate was diluted to 0.3 M NaCl with water, unlabeled dsRNA was added as a carrier, and 3 volumes of ethanol was added to precipitate the dsRNA, which was finally dissolved in 0.3 M STE buffer. Small samples from the final fractions of ^{3}H-labeled ssRNA and dsRNA were digested with 10 μg of RNase per ml (at 0.3 M concentration of NaCl) for 1 h, and the fraction of trichloroacetic acid-precipitable ^{3}H-labeled material was then determined.

This gave an estimate of the relative amounts of ^{3}H-labeled ssRNA and ^{3}H-labeled dsRNA in the two fractions.

Preparation of viral cores. Conversion of purified virions to cores was required both for the analysis of the fraction of defective virions in a population and to obtain standard and defective cores for in vitro transcriptase assay. The use of chymotrypsin for this purpose and the separation of cores by isopycnic centrifugation in CsCl have been described (27). For the transcriptase assay, standard and defective cores, from purified passage 13 R_1 virus, were removed from the CsCl gradients by side puncture of the centrifuge tubes and dialyzed against 0.1 M Tris-hydrochloride buffer (pH 8.0), and the number of particles was determined from the optical density at 260 nm of the respective suspensions (1 optical density unit at 260 nm is equivalent to 6.5×10^{12} standard cores/ml [22] or 7.3×10^{12} defective cores/ml).

Transcriptase assay. Standard and defective cores were used in the transcriptase assay in the amount of 3.3×10^{13} cores/reaction mixture. In a volume of 4 ml the reaction mixture contained 15 μmol of $MgCl_2$, 4 μmol of Tris-hydrochloride, pH 8.0, 20 μmol of phosphoenol pyruvate, 200 μg of pyruvate kinase, 100 μg of purified Bentonite, 1 mmol (200 μCi) of [^{3}H]UTP, and 3 mmol each of ATP, CTP, and GTP. During an incubation period of 8 h at 37 C, approximately 0.2 mg of ssRNA was synthesized, containing 3×10^6 dpm of ^{3}H. The mixture was then centrifuged at 40,000 rpm for 1 h at 4 C in an SW50.1 rotor to remove the viral cores, and the supernatant was made 0.3 M with NaCl and 0.5% with SDS and extracted with phenol. Three volumes of ethanol was added to the resulting aqueous solution, and the mixture was left at 20 C for 18 h. The RNA thus precipitated was centrifuged, washed three times with 90% ethanol, dissolved in 0.05 M sodium acetate, pH 5.3, passed through a column of Sephadex G-100, precipitated with ethanol, and dried in a current of air. If this material was to be analyzed by rate zonal centrifugation, it was dissolved in 0.05 M acetate buffer, pH 5.3, heated to 70 C for 2 min to reduce aggregation, and layered over 17 ml of a 15 to 30% sucrose gradient containing 0.05 M acetate buffer, pH 5.3, and centrifuged at 25,000 rpm for 24 h at 20 C in an SW27.1 rotor.

Hybridization of ssRNA and analysis of the hybrids by PAGE. Transcription products synthesized in vitro were hybridized as follows. Sufficient ^{14}C-labeled dsRNA extracted from purified R_2 virus was mixed with ^{3}H-labeled transcriptase product in 0.01 M STE to give a ratio of dsRNA/ssRNA greater than 50 at a final concentration of approximately 100 μg of RNA per ml. Nine volumes of Me_2SO was added and, after 1 h at 37 C to denature the dsRNA, 3 volumes of ethanol was added to precipitate the RNA. The precipitate was washed three times with ethanol to remove Me_2SO and then dissolved in 1 ml of 0.3 M STE buffer and incubated for 18 h at 72.5 C. The annealed products were precipitated with ethanol, redissolved in 0.3 M STE buffer, made 1 M with NaCl, and left at 4 C for 18 h to precipitate any unhybridized ssRNA and incomplete hybrids. After centrifuging the mixture the supernatant was diluted with 3 volumes of distilled water to reduce the NaCl concentration, and 3 volumes of ethanol was added to precipitate the complete hybrids. Approximately 80% of both the ^{3}H- and ^{14}C-labeled RNA were recovered in this precipitate, which was analyzed by polyacrylamide gel electrophoresis (PAGE) in the manner previously described (27).

Virus-induced ^{3}H-labeled ssRNA extracted from infected cells was hybridized with ^{14}C-labeled dsRNA as described above. The hybridization mixture, however, contained up to 0.5 mg of unlabeled rRNA that had been coextracted from the cells. As shown previously (28) the rRNA did not affect the course of hybridization, and it was finally almost completely removed from the mixture by the precipitation step with 1 M NaCl just prior to PAGE.

RESULTS

Uncoating of defective reovirions in infected cells. In previous work it was found that soon after infection there was a selective transcription of several genomic segments and then a rapid transition to the state where all segments were transcribed with equal frequencies (15). The prime purpose of the present work was to determine the transcription pattern of defective virions lacking the L_1 segment, with the possibility in mind that deletion of this segment might modify the transcriptional control observed in infection with wild-type virus. Isolation of defective virions has been described, and it has been shown that they absorb to L cells at the same rate as standard virus (27). The first experiment in the present series was designed to find whether defective virions are uncoated in the cells after their adsorption.

Infectious virus is probably uncoated in the lysosomes of L cells (23), leading to the formation of subviral particles (SVP) with a distinctive buoyant density in CsCl (24). To find whether defective virions undergo a similar process, L cells were infected with purified R_1d (L_1) virus labeled with ^{32}P. After 3 h at 37 C a cytoplasmic extract was prepared as described above. To this extract was added purified, ^{3}H-labeled R_2 virus to act as a density marker, and the mixture was centrifuged to equilibrium in a CsCl gradient. The results are shown in Fig. 1. The leading peak of ^{32}P represents SVP derived from defective virions. These have approximately the same density, 1.40 g/ml, previously found for SVP formed from infectious virions in infected cells (24). Infectious virus bands at a density of 1.37 g/ml, shown by the trailing peak and defective virions band at the same density (16). It is concluded that the uncoating of defective virus and conversion to SVP proceed in the same manner as with infectious virions.

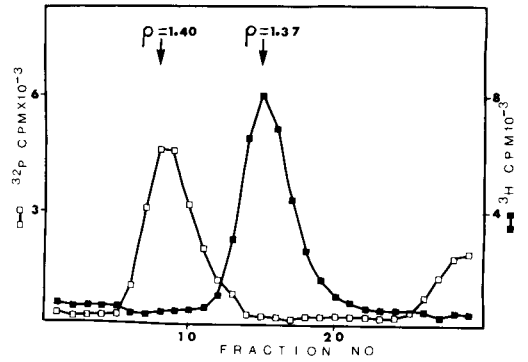

FIG. 1. *Isopycnic centrifugation in CsCl of SVP formed by the uncoating of ^{32}P-labeled R_1d (L_1) virions after adsorption to L cells. ^{3}H-labeled R_2 virions were used as the density marker.*

Transcription of the defective genome. (i) Sucrose gradient sedimentation analysis of transcripts. To determine the transcription pattern of the defective viral genome in vivo, L cells were infected at 37 C with 1,000 R_1d (L_1) virions/cell. Actinomycin D was added at zero time postinfection (p.i.) to suppress cellular RNA synthesis. Virus-specific RNA formed in these cells was labeled with [^{3}H]uridine during three different intervals: 2 to 5, 5 to 10, and 10 to 15 h p.i. At the end of each labeling period the RNA was extracted and analyzed by sedimentation on sucrose gradients. The results are shown in Fig. 2, where the closed squares represent trichloroacetic acid-insoluble RNA labeled during the different intervals.

During the 2- to 5-h period p.i., approximately equal amounts of the *m* and *s* classes of viral RNA were formed, but very little of the *l* class was formed. During the two succeeding periods of labeling, there was a relatively large increase in the amount of *l* class ssRNA synthesized. At no time was there synthesis of viral dsRNA, as shown by the open squares in Fig. 2. These results indicate that during the course of infection with R_1d (L_1) virions the amounts of *l*, *m*, and *s* transcripts change with respect to each other and that deletion of the L_1 segment has prevented replication of the viral dsRNA genome.

(ii) Analysis of the transcripts by hybridization and PAGE. To characterize further the ^{3}H-labeled transcripts represented in Fig. 2, each of the three samples of ssRNA was hybridized with ^{14}C-labeled dsRNA, which had been extracted from purified R_2 virus. The hybrids were analyzed by PAGE (Fig. 3).

In each analysis the 10 genomic segments are well resolved from each other with the exception of L_2 and L_3. Clearly the major hybrids in the 2- to 5-h sample correspond to segments M_3, S_3, and S_4, and very little ssRNA is hybridized with the other dsRNA segments. All segments were transcribed during the 5- to 10-h labeling period, except L_1, but transcripts corresponding to M_3, S_3, and S_4 still predominated. Approximately equal amounts of all hybrids were found with the ssRNA that had been labeled between 10 to 15 h.

A quantitative interpretation can be put on the profiles of Fig. 3. There is known to be a considerable difference in the efficiencies with which the segments of various sizes hybridize with their transcripts (28). In the experiment of Fig. 3 the ^{14}C-labeled dsRNA had been sub-

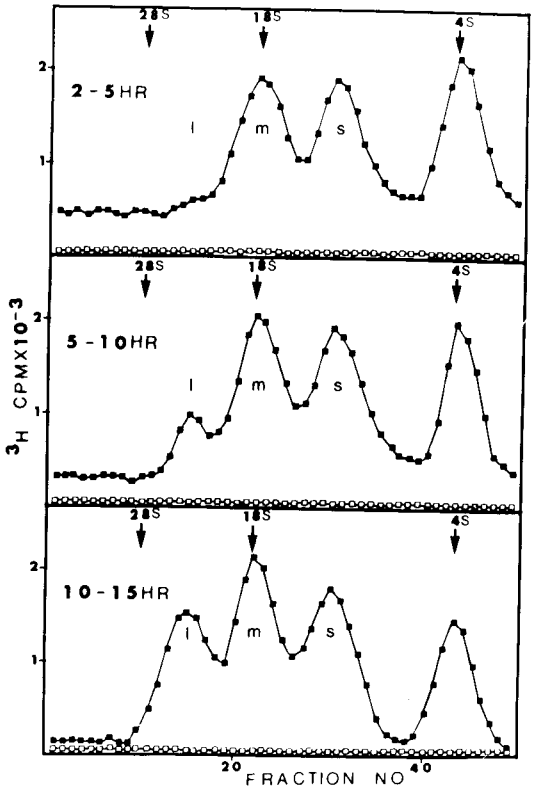

FIG. 2. *Sucrose gradient sedimentation analysis of ^{3}H-labeled RNA formed in cells infected with R_1d (L_1) virions. (Upper) Infected cells labeled between 2 to 5 h p.i. with [^{3}H]uridine in the presence of 0.5 μg of actinomycin D per ml; (middle) labeled between 5 to 10 h p.i.; (lower) labeled between 10 to 15 h p.i. Arrows represent the positions of 28S and 18S rRNA and 4S RNA from the cells. Symbols: (■) trichloroacetic acid-precipitable ^{3}H in each fraction; (□) trichloroacetic acid precipitable ^{3}H after treatment of each fraction with 10 μg of RNase per ml at 37 C for 1 h. l,m, and s represent the three size classes of reoviral ssRNA. Direction of sedimentation is from right to left.*

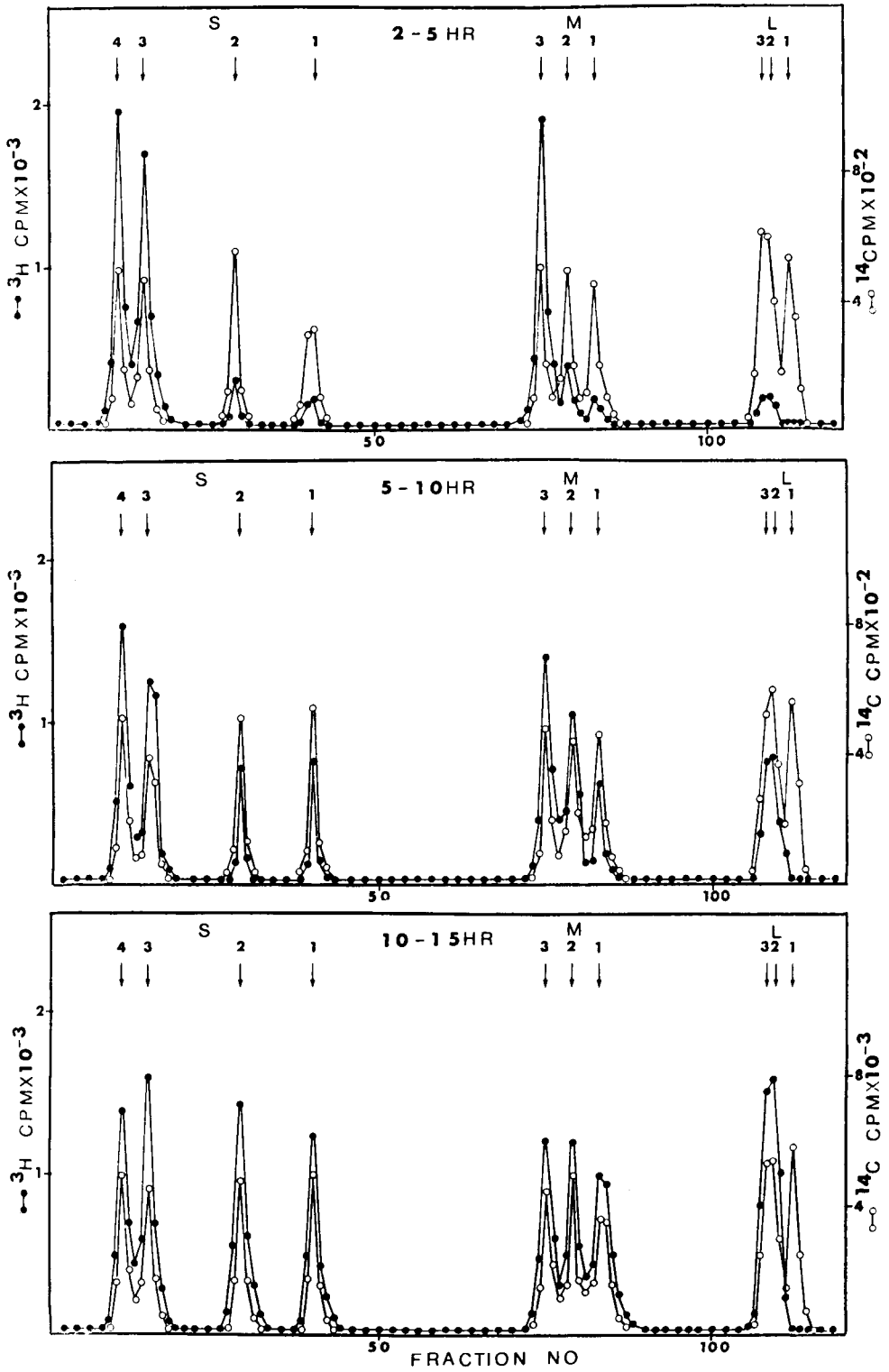

FIG. 3. *PAGE analysis of the hybrids formed between ^{14}C-labeled dsRNA and ^{3}H-labeled ssRNA formed in cells infected with R_1d (L_1) virions. (Upper) Infected cells labeled between 2 to 5 h p.i.; (middle) labeled between 5 to 10 h p.i.; (lower) labeled between 10 to 15 h p.i. Symbols: (●) ^{3}H-labeled ssRNA hybridized with dsRNA; (○) ^{14}C-labeled dsRNA segments. L, M, and S represent, respectively, the large, medium, and small class sizes of genomic segments. Direction of migration is from right to left.*

jected to the denaturation-renaturation process of hybridization, and its presence can thus be used to correct for the differing efficiencies of hybridization of the segments (15). Thus, the $^3H/^{14}C$ ratios of the various peaks in any one gel give the relative frequencies with which the segments had been transcribed in the infected cells. In Table 1 these relative frequencies are shown for the ssRNA synthesized during the three periods of labeling. The results from each gel have been normalized to a value of 10 for segment M_3 as was done in previous work (15). It is evident that between 2 to 5 h p.i. segments S_4, S_3, and M_3 are far more frequently transcribed than the other segments. This pattern is equivalent to the early transcription pattern found with wild-type virus (15, 28), except that the L_1 segment is missing from defective virions and there is no corresponding transcript. During the 10- to 15-h period p.i., all the segments are transcribed with the same frequency, as was found for wild-type virus (15). The 5- to 10-h results show the transition between early and late transcription patterns.

An estimate was made of the amount of viral RNA formed in cells infected with defective virions. Parallel cultures were infected with 1,000 defective virions/cell, or 20 PFU of wild-type virus per cell, and actinomycin D was added at zero time p.i. Both cultures were labeled with [^{3}H]uridine between 2 to 15 h p.i., and the RNA of the cells was then extracted with SDS-phenol and passed through a Sephadex G-100 column as described in Materials and Methods. The resulting virus-specific ssRNA obtained from cells infected with defective virus was approximately 3% of that found for the infection with wild-type virus.

Two conclusions are drawn from these results. First, the L_1 function is required for the replication of viral dsRNA. Second, transcription of the defective viral genome in infected cells is subject to the same kind of regulatory process as the wild-type genome. Consequently, the L_1 function is not involved in "turning on" transcription of the late segments of the genome.

TABLE 1. *Relative frequencies of transcription of the genomic segments in cells infected with R_1d (L_1) virions*

Genomic segment	$^3H/^{14}C$ in the hybrids[a]		
	2-5 h[b]	5-10 h	10-15 h
L_1	0.1	0.1	0.1
L_2	1.0	4.2	8.6
L_3	1.0	4.2	8.6
M_1	0.9	2.5	8.2
M_2	1.6	6.5	9.8
M_3	10.0	10.0	10.0
S_1	1.2	3.7	8.4
S_2	1.4	3.9	9.8
S_3	9.6	10.0	9.8
S_4	9.8	11.0	9.0

[a] $^3H/^{14}C$ ratios were determined from the profiles of Fig. 3 and normalized to a value of 10 for segment M_3.

[b] Labeling period.

Transcription of the genome of ts mutant R_2C (447) at the nonpermissive temperature. Since we have recently provided evidence that the C class *ts* mutation is in the L_1 segment of the viral genome (26) it would be predicted that, in cells infected at the nonpermissive temperature with this mutant, one should see a regulated transcription of the genome similar to that found with the defective virions. One major problem arises with this mutant, however, since it has been shown to give rise to defective virions with one or more segments deleted (18). We have confirmed this report that such deletions can occur after only the third to fourth passage of a picked plaque of the mutant. Consequently, most populations of the C mutant contain defectives that could readily give a complex transcription pattern in cells infected with them. We have proceeded in the following way to get a preparation of the C mutant free from defective virus.

Two cultures of L cells were infected with fourth-passage C mutant, one at an MOI of 0.1 PFU/cell and the other at an MOI of 2 PFU/cell. [^{3}H]uridine was added, the cultures were placed at 31 C for 40 h, and the cells were then collected and the viral yields from each culture were purified. Each purified preparation was tested for the presence of defective virus. To do this a small sample of the preparation of C mutant was mixed with ^{14}C-labeled standard R_2 virions, and the mixture was treated with chymotrypsin and then subjected to isopycnic centrifugation in CsCl. The results with progeny virus obtained from a culture infected with 2 PFU/cell are shown in Fig. 4b. The leading peak of ρ = 1.43 g/ml represents viral cores derived from complete C mutant virions cosedimenting with cores derived from the wild-type R_2 virions. A trailing peak at ρ = 1.42 represents the defective virions derived from the C mutant, and it is seen that in this preparation over 30% of the virion population was defective. A similar analysis is shown in Fig. 4a for virus obtained from the culture infected at MOI = 0.1 PFU/cell. Virtually all the C mutant cores cosedimented with cores from the marker R_2 virus, and thus this preparation is practically free of defective virus. A similar unlabeled, defective-free preparation was then used in the following experiments to examine transcription

in cells infected with C mutant.

Two cultures of L cells were infected at an MOI of 20 PFU/cell with the C mutant preparation and placed at 39 C. One culture was labeled with [^{3}H]uridine between 2 to 5 h p.i. and the other between 8 to 11 h p.i. After each labeling period, the ssRNA was isolated and a small sample was analyzed by sucrose gradient sedimentation. All three size classes of viral ssRNA were synthesized during both intervals (data not shown) but, as found for the defective virions (Fig. 2), there was no synthesis of viral dsRNA. To characterize the transcripts further, they were hybridized with ^{14}C-labeled dsRNA from purified R_2 virions and analyzed by PAGE. The results are shown in Fig. 5 and 6. ^{3}H/^{14}C ratios were calculated for each peak in the two gels of Fig. 6 and entered in Table 2. Between 8 to 11 h, all segments were being transcribed with equal frequencies, similar to the late transcription pattern seen with wild-type virus (15) and defective virions (Table 1). However, between 2 to 5 h, segments L_1, M_3, S_3, and S_4 were transcribed with higher frequencies than the remaining segments, although the difference was not as marked as that found with defective virions during the same period (Table 1). Thus in cells infected with either the C class mutant, defective virions, or wild-type virus there is a selective transcription of the same segments at early times and then a transition to a situation in which all segments are

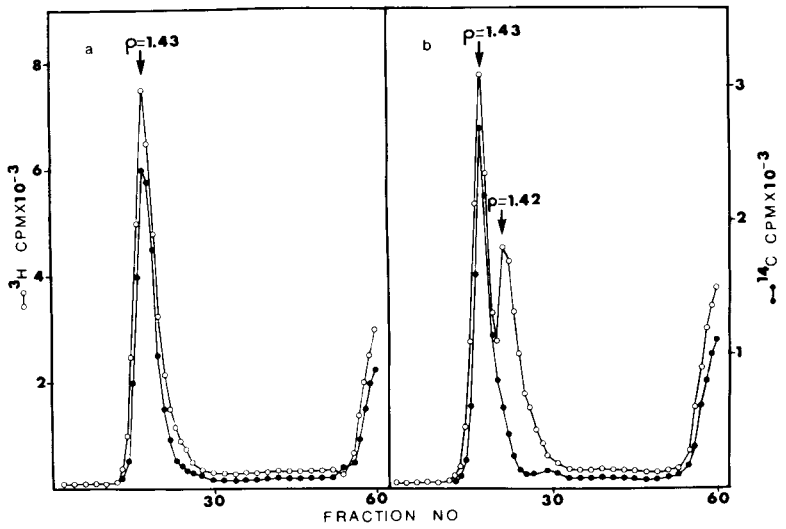

FIG. 4. *Isopycnic centrifugation in CsCl of the chymotrypsin-derived cores from C mutant virus. Purified ^{14}C-labeled R_2 virions were added to each preparation of C mutant prior to chymotrypsin treatment to act as a density marker. (a) Purified viral progeny obtained from a culture infected with C mutant at MOI = 0.1 PFU/cell; (b) progeny obtained from infection with the same C mutant preparation at MOI = 2 PFU/cell.*

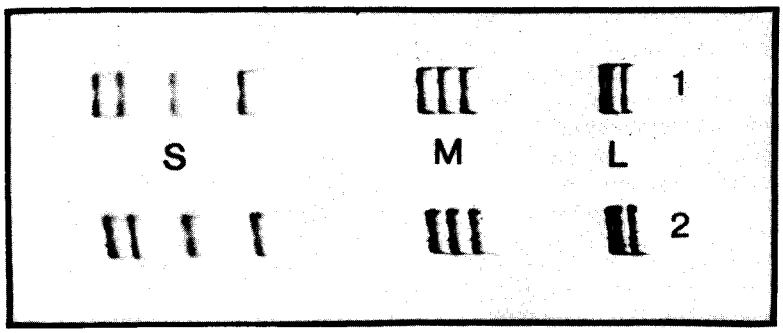

FIG. 5. *PAGE analysis of the hybrids formed between ^{14}C-labeled dsRNA obtained from standard virions and ^{3}H-labeled ssRNA formed in L cells infected with R_2C (447) at 39 C. The two patterns are autoradiograms of hybrids formed (1) from ssRNA synthesized 2 to 5 h p.i. (2) ssRNA synthesized 8 to 11 h p.i. The bands are formed by exposure of the film to the ^{14}C in the marker dsRNA.*

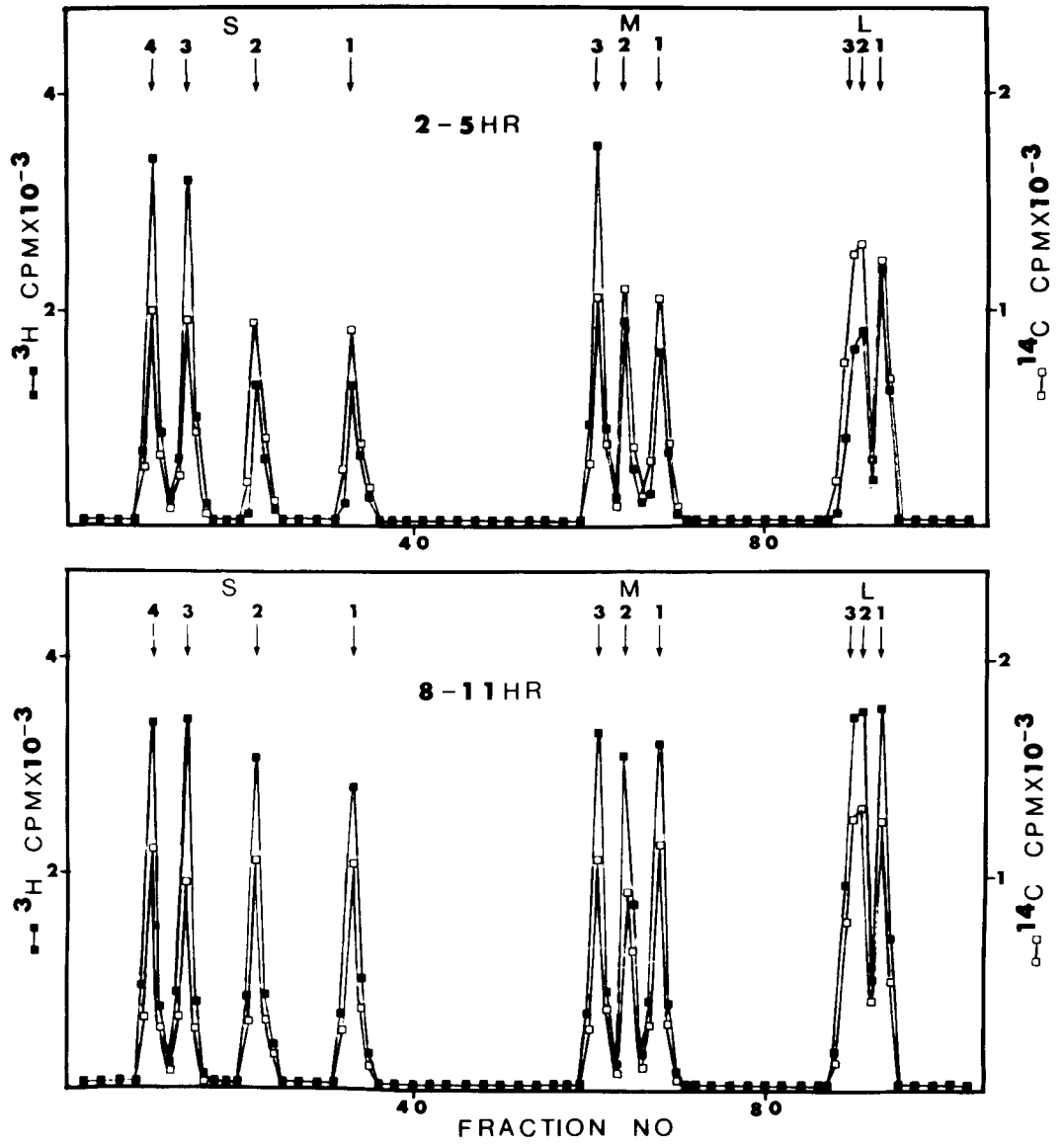

FIG. 6. *PAGE analysis of the hybrids formed between ^{14}C-labeled dsRNA and ^{3}H-labeled ssRNA synthesized in cells infected with R_2C (447) virus at 39 C. (Upper) Infected cells labeled between 2 to 5 h p.i.; (lower) infected cells labeled between 8 to 11 h p.i. Quantitative assay by scintillation counting of ^{14}C and ^{3}H in slices of the two gels which are shown in Fig. 5.*

transcribed with equal frequencies.

The amount of virus-specific ssRNA found in cells infected with the C mutant was determined in the same way as described for the defective virions in a previous section. In the period 2 to 15 h p.i. at 39 C the amount of RNA synthesized was approximately 5% of that formed in cells infected with wild-type virus, as had been found previously (9).

In vitro synthesis of ssRNA with standard and defective cores. Having observed the regulated transcription of the defective viral genome in vivo, it was of interest to determine the transcription pattern in vitro. For this purpose equal numbers of standard and defective cores derived from R_1 virus were added separately to transcriptase reaction mixtures containing [^{3}H]UTP, as described in Materials and Methods. Samples were withdrawn at intervals from each reaction mixture and assayed for trichlo-

TABLE 2. *Relative frequencies of transcription of the genomic segments in cells infected with R_2C (447) virus at 39 C*

Genomic segments	$^3H/^{14}C$ in the hybrids[a]	
	2-5 h[b]	8-11 h
L_1	6.5	9.6
L_2	4.0	9.3
L_3	4.0	9.3
M_1	4.9	9.5
M_2	5.4	11.0
M_3	10.0	10.0
S_1	4.7	9.0
S_2	4.2	9.3
S_3	9.6	11.0
S_4	10.0	9.8

[a] $^3H/^{14}C$ ratios were determined from the profiles of Fig. 6 and normalized to a value of 10 for segment M_3.

[b] Labeling period.

roacetic acid-insoluble ^3H-labeled ssRNA. The kinetics of the reactions determined in this way are shown in Fig. 7. The amount of ssRNA formed with defective cores was 80 to 85% of that synthesized with standard cores.

An in vitro reaction was carried out for 8 h, and an analysis by sucrose gradient sedimentation (not shown here) indicated that the *l* class of transcript formed from defective cores was considerably less than that formed with standard cores. The two reaction products were therefore further examined by hybridizing ^{14}C-labeled dsRNA with the ^3H-labeled transcripts and analyzing the hybrids by PAGE. The results are shown in Fig. 8, and the $^3H/^{14}C$ ratios for each peak are given in Table 3. As expected, no transcript was formed from the L_1 segment of the defective genome; the remaining segments were transcribed with the same relative frequencies as the standard genome. That is, for the defective genome the relative frequencies of transcription of the remaining segments are inversely proportional to their molecular weights, as has been shown previously for standard cores (25).

DISCUSSION

At early times after infection with wild-type reovirus there is a selective transcription of four genomic segments, L_1, M_3, S_3, and S_4 (early transcription pattern), and then a transition to a situation in which all 10 segments are transcribed with equal frequencies (late transcription pattern) and the genome is replicated (15, 28). This process, which is normally asynchronous in a mass culture, can be divided into several sequential steps. If the cells are infected in the presence of cycloheximide, the four early segments L_1, M_3, S_3, and S_4 are predominantly transcribed and the genome is not replicated. Apparently one or more of the four early gene products is involved in turning on transcription of the rest of the genome. Another stage in the process is shown by infecting cells with the deletion mutant R_1d (L_1) or with the class C *ts* mutant at nonpermissive temperature as we have done in the present work. Under these conditions the four early segments are selectively transcribed, three segments in the case of defective virus because L_1 is missing from it, the remaining segments are then turned on, and, finally, all segments are transcribed with equal frequencies. The genome is not replicated with either mutant.

Thus, in contrast to many other viral systems, the late pattern of transcription is found with reovirus prior to genome replication. In fact, these transcripts are used as templates for the synthesis of complementary strands to form the dsRNA segments of the progeny genomes (17). Since the parental genome remains firmly associated with parental virion structures (SVP) throughout the course of infection (24)

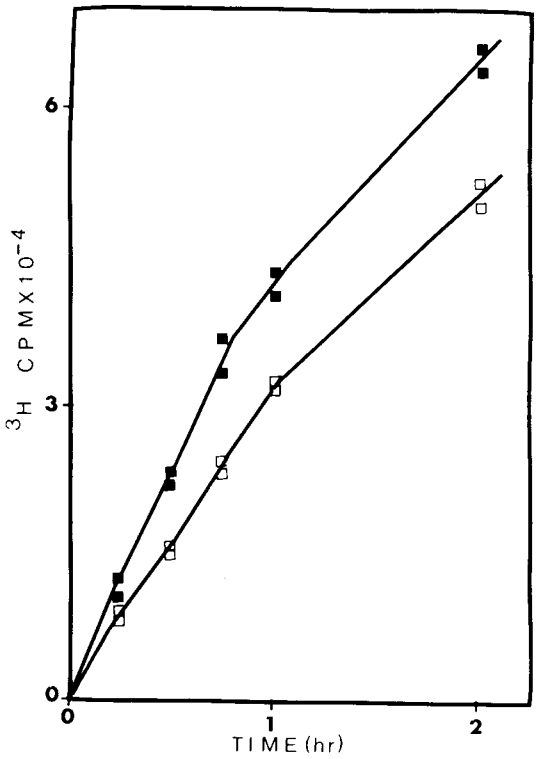

FIG. 7. *Kinetics of synthesis of ssRNA formed in vitro with chymotrypsin-derived viral cores. Formation of trichloroacetic acid-insoluble RNA in reaction mixtures containing 3.3 × 10^{13} standard cores (■) or the same number of defective cores (□).*

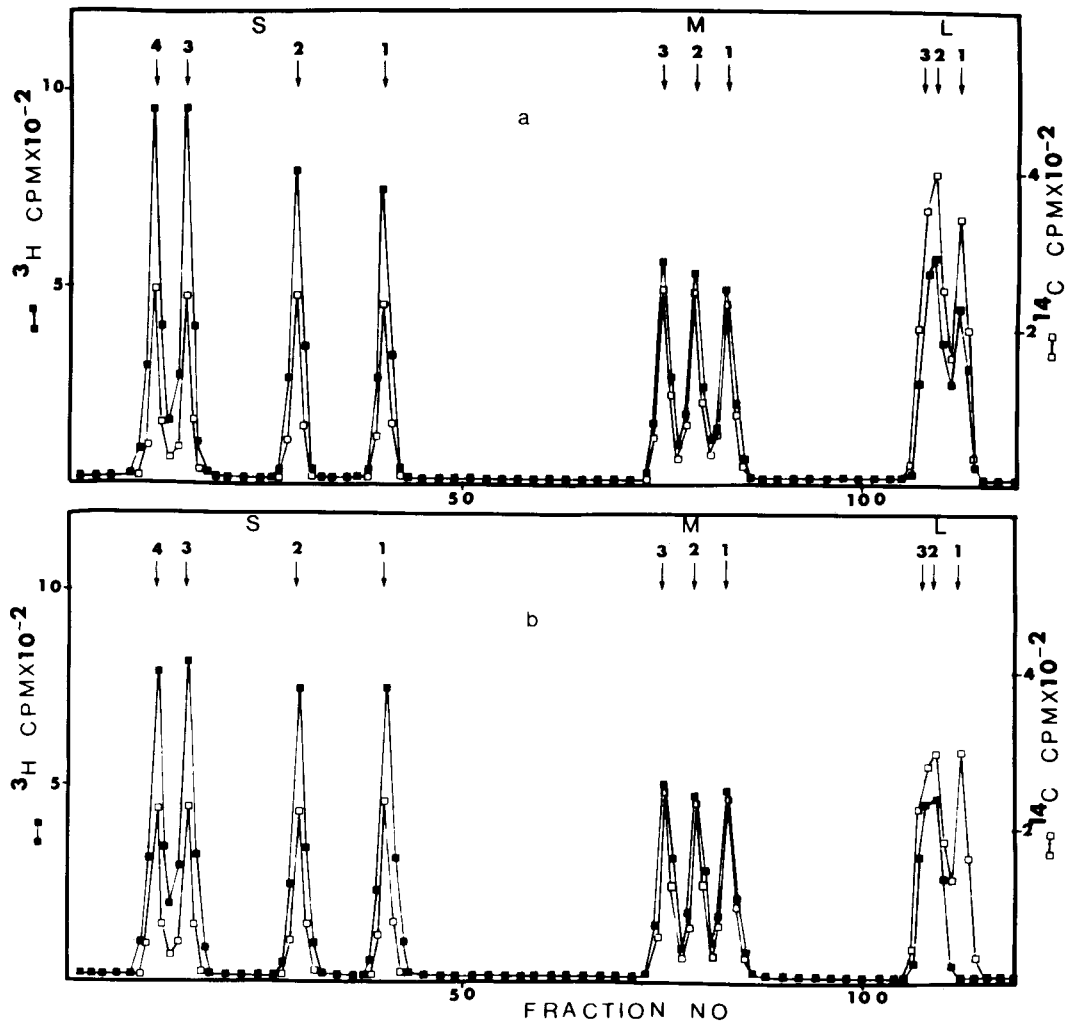

FIG. 8. *PAGE analysis of the hybrids formed between ^{14}C-labeled dsRNA and ^{3}H-labeled ssRNA formed in vitro with viral cores. (Upper) Synthesis with standard viral cores; (lower) synthesis with defective cores.*

and is never free in the cell (8), its transcription must be carried out by the RNA polymerase, which is an integral constituent of the infecting virus (1, 21). Indeed, the observation, which we have confirmed, that the recombination frequencies between *ts* mutants do not increase during the replicative cycle (3) suggests that all progeny genomes are formed with transcripts from the parental genome. Regulation of transcription in this system therefore implies a control on the action of the infecting virion polymerase, since after replication of the genome all segments of the progeny genomes continue to be transcribed with equal frequencies (15).

If an early gene product(s) is involved in turning on late transcription of the parental genome one might expect to find a class of *ts* mutants that does not permit progression beyond early transcription at the nonpermissive temperature. So far no such mutant has been found. Of the present classes of dsRNA$^-$ mutants only two appear to carry the *ts* mutation in early segments. Class C has the mutation in the L_1 segment (26), and the class E mutation is probably in the S_3 segment (18). The D mutation may be in the M_2 segment (18), which does not code for an early function. Under nonpermissive conditions all segments of these three classes of mutants are transcribed in infected cells (2, 9). Moreover, all the normally occurring viral polypeptides are also found (5, 6), indicating that the transcripts may serve a dual function as mRNA's as well as precursors of the progeny dsRNA genomes. The three classes

of dsRNA$^-$ mutations thus appear to be in functions involved in genome replication rather than in control of transcription. We suggest therefore that mutations are still to be found in the early functions coded by segments M_3 and S_4 and that either one or both of these functions might control the transition from early to late transcription of the parental genome. One might expect to find a further class of mutants with a temperature-sensitive virion transcriptase, in which case there should be no transcription of the parental genome under nonpermissive conditions. The transcriptase of the class G mutant is somewhat temperature sensitive in in vitro tests, but this is not the major *ts* lesion in the G mutant since the virion polymerase should act as a *cis*-function and yet the G mutant can readily complement the growth of the defective virions, R_1d (L_1), at the nonpermissive temperature (26). Presently seven classes of *ts* mutants are known, each class thought to represent lesions in one or other specific segment of the genome. If this is the case three classes of mutants remain to be discovered and, as we have suggested above, some prediction can be made about the phenotype of at least two of these classes.

The clearest demonstration of a regulated transcription in this system is obtained by the use of cycloheximide added at the time of infection to inhibit protein synthesis (14, 15, 28). Under these conditions the predominant transcription is from four segments and this result has been confirmed (9). However, regulated transcription without the use of cycloheximide has not been found in other laboratories (2, 9), and the question has arisen whether the transcription pattern found with cycloheximide might be an artifact. We have recently shown that the early transcripts formed in cycloheximide-treated cells are functional mRNA's, which seemed to remove this objection (12). Joklik has suggested further (11) that when the overall rate of transcription is low, as early transcription always is, those transcripts formed in least amount might be lost during manipulations involved in their assay and thus one might gain the illusion that only four segments were transcribed. Indeed, inspection of the PAGE patterns (reference 14, Fig. 4; reference 15, Fig. 1) suggests that transcription in cycloheximide cells may not be confined exclusively to four segments; perhaps L_2 is also transcribed to a limited extent, but in this region of the gels resolution of the hybrids is not good enough to be certain.

Nevertheless, we have consistently found an early-late regulation of transcription in wild-type reovirus infection (15, 28). The use of cycloheximide merely accentuates the early transcription pattern, which can be seen without using the inhibitor. In the present work a regulated transcription of two mutant genomes has been described similar to that observed with wild-type virus. Other laboratories have not found a distinctive early transcription with wild-type virus, the C mutant, or with the D and E mutants (2, 9), which might be expected to behave similarly to the C mutant in this respect. The explanation of these differences may well lie in the techniques used by the different laboratories to quantitate the transcripts. Viral ssRNA is assayed by labeling it with a radioactive precursor, hybridizing to denatured viral dsRNA, and analyzing the resulting hybrids by PAGE. As expected, we have found that the efficiency of forming hybrids between the transcripts and their respective segments varies with the molecular weight of the segment (28). Consequently, we have invariably standardized our PAGE analyses by hybridizing ^{3}H-labeled transcripts with a large excess of ^{14}C-labeled viral dsRNA. In any given gel analysis the ^{3}H/^{14}C ratios determine directly the relative frequencies with which the segments have been transcribed, automatically correcting for the varying efficiencies of hybridization of the segments (15). Other laboratories have generally quantitated their gel analyses of hybrids through radioautography, using the single label in the transcript. Without the double label it seems possible that the hybridization technique is not sufficiently sensitive to pick up the changing frequencies of transcription that we find early in the infectious process.

No regulation corresponding to the in vivo situation has been observed during the in vitro transcription of the viral genome (25). All 10

TABLE 3. *Relative frequencies of transcription in vitro of the genomic segments in cores of R_1 and R_1d (L_1) virus*

Genomic segment	^{3}H/^{14}C in the hybrids[a]	
	R_1 cores	R_1d (L_1) cores
L_1	5.7	0.2
L_2	6.1	6.1
L_3	6.1	6.1
M_1	8.9	9.1
M_2	9.4	9.5
M_3	10	10
S_1	15	16
S_2	16	17
S_3	18	19
S_4	19	19

[a] ^{3}H/^{14}C ratios were determined from the profiles of Fig. 8 and normalized to a value of 10 for segment M_3.

segments are transcribed whether the transcriptase is provided by cores formed from virions by extensive digestion with chymotrypsin (25), by SVP obtained from infected cells (24), or by SVP (SVP_i) produced by limited chymotrypsin action on virions (21). The latter particles (SVP_i) are of particular interest since they are similar in composition to SVP formed when virions are uncoated in infected cells and largely retain the infectivity of the original virion population. When cells are infected with SVP_i in the presence of cycloheximide the four early segments, L_1, M_3, S_3, and S_4, are the predominant ones transcribed (20). In the absence of cycloheximide all 10 segments are transcribed. Whether there was a selective early transcription in the absence of cycloheximide was not determined. Nevertheless, this experiment strongly supports the idea (15) that a protein of cellular origin is involved in turning on transcription of the late segments. We might suppose that when the virion is uncoated in the infected cells transcription of the early segments commences rapidly, but the remaining six segments are repressed by the hypothetical cellular protein. One of the early viral gene products might then combine with this cellular protein to derepress transcription of the remaining segments. The use of mutants temperature sensitive in the derepression step might be the simplest way to test this hypothesis.

Two basic models might be postulated for the mechanism of transcription of the reovirus genome. In the first model, the assumption is made that the dsRNA segments are arranged (perhaps linked) in such a fashion that a polymerase molecule initiates transcription at one end of the genome and traverses the entire genome. A complete copy of the genome is thus synthesized per unit time. In this coordinate model, the transcription product would contain an equal number of copies of each genome segment. If the transcripts were labeled with ^3H and hybridized with ^{14}C-labeled dsRNA, PAGE analysis of the hybrids would show a constant $^3H/^{14}C$ ratio, indicating a uniform frequency of transcription for all segments. This is the result we have found at late times after infection of cells with wild-type virus (15) and in the present work, at late times after infection with the C mutant at nonpermissive temperature or with defective virions.

The second model assumes that each segment is copied independently of the others, that each segment must be transcribed completely before the next transcript is initiated, and that the rate of nucleotide addition of the transcripts is constant for all segments. In this event, the amount of each transcript made in unit time (or frequency of transcription) would be inversely proportional to the molecular weight of the segment. This will be termed the independent model, and the results obtained here for the in vitro transcription of both standard and defective cores are consistent with this model. Moreover, according to the coordinate model, one might expect that the amount of ssRNA synthesized per unit time in vitro would be the same whether the largest segment was present in the viral cores or not. The independent model predicts that approximately 15% less ssRNA would be synthesized by defective cores in vitro than by an equivalent number of standard cores, and this was the result found (Fig. 7). Further evidence in favor of this independent model for in vitro transcription is suggested by the observation that up to nine different RNA strands have been seen extruded from cores actively synthesizing ssRNA transcripts (7).

At face value, therefore, transcription of the reoviral genome can be explained by two quite different mechanisms, depending on the circumstances of transcription, and possibly both mechanisms are utilized. It is, however, simpler to imagine that the completely unregulated transcription of the genome by the virion polymerase working at its maximum rate in vitro is accurately described by the independent model and that the segments are transcribed independently in vivo as well. If so, some control must be placed on the transcription of each segment to give the early pattern of transcription changing to the late pattern during the course of infection. There are a number of ways in which independent transcription might be modified to give the appearance of a late coordinate transcription. Transcription could be regulated by virus- or cell-coded repressors or even by a limiting supply of nucleoside triphosphates at the site of ssRNA synthesis. In fact, one merely needs to assume that late in infection each segment can be transcribed by more than one transcriptase molecule at any given time to simulate the late pattern we have obtained. Whether any of these rather speculative schemes have any basis in reality must be left for future work to determine.

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