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Molecular Biology of Rotaviruses VIII. Quantitative Analysis of Regulation of Gene Expression during Virus Replication

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A sensitive and quantitative solution hybridization assay recently developed in this laboratory has been applied to the study of the regulation of viral gene expression in rotavirus-infected cells. Measurement of the cumulative level of viral plus-strand (mRNA) synthesis at hourly intervals throughout the growth cycle has provided evidence for both quantitative and qualitative regulation of transcription. Qualitative control was found only when cycloheximide was used to block protein synthesis in infected cells, when transcription of four of the viral genes (genes 5, 6, 7, and 9) was independent of protein synthesis. Quantitative regulation was demonstrated by the accumulation of mRNA to much higher levels for some of the viral genes (e.g., genes 2 and 7) relative to others (e.g., genes 4 and 6). In addition to quantitative control at the level of transcription, measurement of the relative molar amounts of the various viral proteins at 6.5 h postinfection showed that their levels did not directly reflect those of their encoding RNAs, indicating the existence of translational control of gene expression in the rotavirus system. Analysis of the levels of minus strand synthesized for each viral gene showed that they were not all accumulated to the same level. The significance of this result in the light of the presumed similarities in replication strategy to that of the mammalian reoviruses is discussed.

It is now some 15 years since rotaviruses were identified as a major cause of acute viral gastroenteritis in the young of humans and all the major species of domestic livestock (5, 7). In the developing countries of Africa, Asia, and Latin America, rotavirus infection is perhaps the major single cause of infant mortality, accounting for an estimated 2 million to 5 million deaths per annum (3, 10). In the so-called developed world, the problems caused by rotavirus infection are mainly economic ones, which can be subdivided into two broad categories. In the agricultural industry, animal wasting and, in severe cases, death as the result of rotavirus-induced diarrhoea are estimated to cost millions of pounds each year in the British dairy industry alone. In humans, a significant amount of the infant morbidity caused by gastroenteritis can be attributed to rotavirus infection, which is costly to treat. Because of the medical and agricultural importance of rotaviruses, there is now a substantial amount of interest in the development of an effective antirotavirus vaccine. To help support advances toward this goal, it is desirable that we should have a more complete understanding of rotavirus replication at the molecular level.

Rotaviruses are members of the family *Reoviridae*, having a genome composed of a number, 11, of discrete segments of double-stranded RNA (dsRNA) (18, 25, 28). The molecular details of their replication cycle have not been analyzed to date, but their similarity in genome type to the mammalian reoviruses, whose replication cycle has been analyzed in some detail, has led to the assumption that rotavirus replication would follow the same basic strategy as that of reovirus. The information available from the reovirus system formed a good basis on which to begin our experimental work. Mammalian reoviruses have a genome composed of 10 segments of dsRNA which have been shown to replicate in a nonconservative fashion (1, 27). The mechanism first involves the transcription of the parental input dsRNA genome within the viral core by a virion-associated RNA polymerase to generate mRNA. This mRNA serves two functions: it is translated to produce the viral encoded proteins, and it also acts as the template for the synthesis of new minus-sense RNA strands to produce progeny genomes, a process that takes place inside a particulate preprogeny virion. The transcription of reovirus RNA was studied some years ago by Nonoyama et al. (20), who used hybridization of in vivo-labeled RNA to an excess of unlabeled genomic RNA to capture the transcripts into dsRNA hybrids and then quantify them by polyacrylamide gel analysis. In addition to the quantitative regulation of transcription, they found qualitative control, since only 4 of the genes were transcribed at early times postinfection, whereas all 10 were found at later times. If infected cells were treated with cycloheximide to block protein synthesis, the early pattern of viral transcription could be preserved until late in infection. This suggests that at least some of the protein products of the four early genes may play a regulatory role in the replication cycle (12). Unfortunately, the technique used by Nonoyama et al. (20) was not able to distinguish between plus-sense mRNA transcripts and new minus-sense RNA copies, and although it allowed the relative amounts of the different transcripts to be compared, it could not do so in terms of absolute numbers of RNA molecules per infected cell.

The aim of our studies with rotavirus was to analyze in detail both viral transcription and replication, and it was therefore important to use an assay capable of distinguishing between newly synthesized plus and minus RNA strands. The assay developed (9) is a solution hybridization which makes use of virus strand-specific probes generated by in vitro transcription of cDNA copies of viral genes from the SP6 and T7 promoters of the Gemini transcription vectors. The assay has been exploited to accurately measure the levels of plus- and minus-strand synthesis for each of the 11 rotavirus genes. In addition, because the complete RNA protein-coding assignments for the UKtc bovine virus are

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known (14), we also aimed to determine the relative translational efficiencies of each of the viral mRNAs by quantifying the levels of the various virus-encoded proteins.

MATERIALS AND METHODS

Cells and virus stocks. BSC-1 cells were maintained in Glasgow modified minimal essential medium supplemented with nonessential amino acids, 5% fetal calf serum, and 50 μg of gentamicin sulfate per ml. The Compton UK tissue culture-adapted bovine rotavirus strain was originally obtained from M. Thouless. Virus stocks were prepared by infection of confluent monolayers of BSC-1 cells at a multiplicity of infection of 0.1 PFU per cell, and growth was allowed to proceed for 72 h at 37°C. The infectivity of the virus stock was determined by titration on BSC-1 cells (13).

Preparation of infected cells and extraction of RNA. The accumulation of both strands of all 11 rotavirus genes was analyzed on the same stock of infected-cell RNA by using 150×10^6 cells contained in two 650-cm^2 roller bottles for each sampling time. The infected-cell RNA was standardized by labeling the cells with [^{3}H]uridine (2.5 μCi/ml) for 16 h prior to infection. At the time of infection, the uridine-containing medium was removed from the cells to ensure labeling of cellular RNA only. Cells were infected at a multiplicity of 10 PFU per cell and harvested by trypsinisation (0.05% trypsin) and low-speed centrifugation at hourly intervals during an 8.5-h infection. Cell pellets were suspended in 10 ml of 50 mM Tris hydrochloride (pH 8.0), and a small sample was reserved for titration of infectivity (13). The remainder was dispersed by sonication followed by 10 strokes with a Dounce homogenizer. Sodium dodecyl sulfate was added to a final concentration of 1%, and the suspension was extracted once with phenol equilibrated with 0.1 M sodium acetate (pH 5.0) at 60°C and twice with water-saturated phenol at 37°C. RNA was collected by precipitation with 2 volumes of absolute ethanol in the presence of 0.15 M lithium chloride and suspended in 1,500 μl of piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-formamide hybridization buffer (40 mM PIPES [pH 6.7], 0.4 M sodium chloride, 1 mM EDTA, 80% deionized formamide). The trichloroacetic acid-precipitable radioactivity (^{3}H) in each sample was then used to adjust the volumes so that all samples contained the same amount of cellular RNA per unit volume.

Production of RNA copies of rotavirus cDNA sequences by in vitro transcription. Rotavirus-specific cDNA clones originally isolated by McCrae and McCorquodale (15) were subcloned into appropriate restriction enzyme sites within the polylinker region of pGEM1 and pGEM2 transcription vectors (Promega Biotec). The size of the cDNA inserted was in the range of 200 to 1,150 base pairs. Small amounts of high-specific-activity RNA and larger amounts of unlabeled RNA were generated from these clones and used in solution hybridization assays as described by Johnson and McCrae (9) to evaluate the accumulation of specific RNA species throughout infection.

Analysis of protein synthesis. Cells were incubated in methionine-free medium for 16 h prior to pulse labeling (at 6.5 h postinfection) with [^{35}S]methionine (800 Ci/mmol). Samples were balanced with respect to trichloroacetic acid-precipitable radioactivity, and labeled polypeptides were fractionated on 5 to 11% polyacrylamide gels by using the Laemmli discontinuous buffer system (11). Treatment with diphenyloxazole allowed fluorography to be carried out. When cycloheximide was used, it was added 2 h prior to infection at a concentration of 20 μg/ml and maintained until indicated.

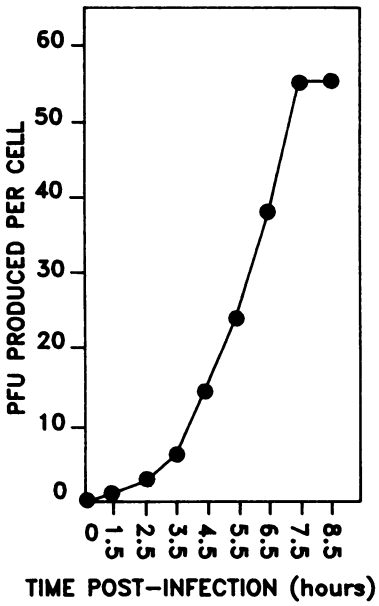

FIG. 1. One-step growth of rotavirus: production of infectious progeny virions. Cells were infected at a multiplicity of 10 PFU per cell and harvested at hourly intervals throughout an 8.5-h growth curve. Titers of samples were found as described in Materials and Methods. The overall yield of virus was calculated from these results to be 54 PFU per cell.

RESULTS

Parameters of the growth cycle used for analysis. To ensure that the results of analysis of the different viral genes could be directly compared, we prepared large quantities of cells sufficient to carry out assays on all the genes for each time point used. In addition to extracting most of the cells for RNA analysis, plaque assays and particle counts were carried out on each sample so that the kinetics of the one-step growth curve under the conditions used could be related to the biochemical results from the RNA studies. Under the conditions of infection used (multiplicity of infection, 10 PFU per cell), the expected one-step growth curve was produced (Fig. 1). After an initial eclipse phase of 3.5 h, progeny virion production proceeded exponentially until 7.5 h postinfection, at which time a plateau was reached. The overall yield of infectious virus during the replication cycle used in these studies was 54 PFU per cell, which represents an approximate fivefold amplification of the input infectivity. A particle-to-PFU ratio was measured for the 8.5-h-postinfection sample and was shown to be 4.7. This illustrated that under the conditions used in the virus plaque assay, only approximately 20% of the progeny virus particles were capable of causing an infection.

Kinetics of synthesis of plus-sense RNA strands (mRNA). A compilation of the data obtained for the accumulation of plus-sense RNA strands (mRNA) for each of the viral genes is shown in Fig. 2. Inspection of these results revealed two levels of transcriptional control. First, there was clear temporal control of synthesis, with three broad patterns of mRNA accumulation being discernible. In the first category (genes 1, 2, 6, 8, and 11), mRNA synthesis proceeded at a more or less linear rate throughout the infection cycle. The

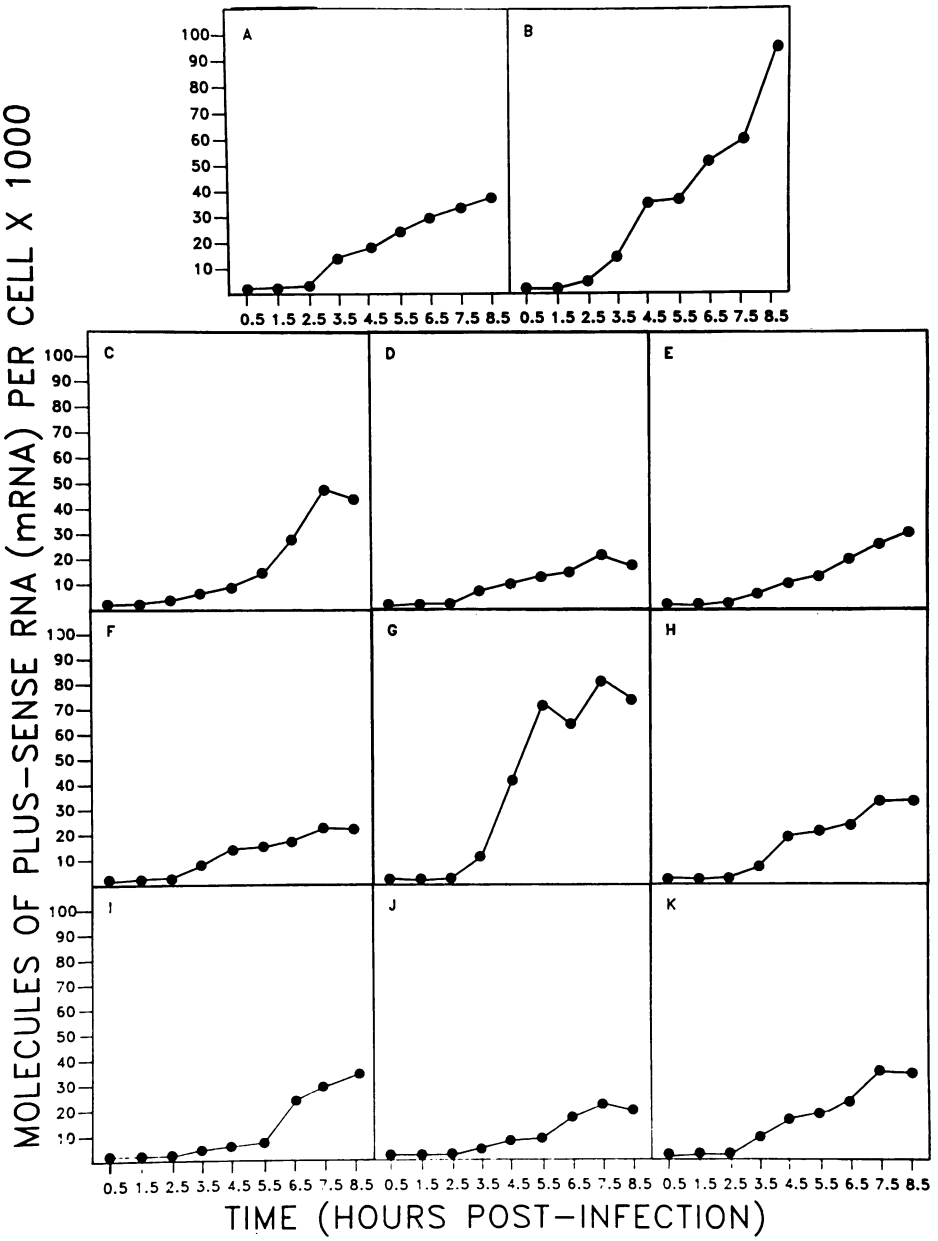

FIG. 2. Accumulation of mRNA. The figure shows the accumulation of plus-sense (mRNA) strands for the 11 rotavirus genes occurring during a single-step growth experiment carried out as described in Materials and Methods. The results obtained for genes 1 to 11 presented as molecules of RNA per cell are shown in panels A to K, respectively.

second pattern was exhibited by only one gene (gene 7), and in this case, mRNA accumulated rapidly during the early part of the infectious cycle and then reached a plateau that was maintained for the remainder of the growth cycle. The third category of genes (genes 3, 4, 5, 9, and 10) contained those in which there was a relatively slow accumulation of mRNA during the early part of the growth cycle, with the rate increasing later. It was not possible in this initial accumulation experiment to see any distinct division or switch between a set of early and late genes in terms of mRNA synthesis. The absolute levels of mRNA accumulating at early times for some genes, e.g., 1, 2, and 7, were higher than for others. However, because of the incomplete inhibition of host protein synthesis at early times postinfection, it was not possible to accurately measure the relative levels of synthesis of the corresponding viral proteins, and hence it was impossible to assess whether the increased mRNA levels resulted in increased protein synthesis.

The second level at which transcriptional control was evident was a quantitative one. Inspection of the data shown in Fig. 2 showed that up to and including 7.5 h postinfection, the viral genes could be divided into four broad categories based on the amount of mRNA accumulated. The highest levels (73,000 to 94,000 molecules per cell) were reached by two genes 2 and 7. Gene 3, which accumulated 45,000 molecules per cell, was the sole representative of the second group. In the third group, which had accumulated 30,000 to 36,000 molecules per cell by 7.5 h postinfection, there were

TABLE 1. Relative production of rotavirus proteins at 6.5 h postinfection[a]

Protein	Relative molar amts synthesized (-fold)
VP1	0.15 ± 0.05
VP2	1.32 ± 0.14
VP3/4	1.27 ± 0.12
VP5	1.57 ± 0.18
VP6	25.02 ± 2.52
VP7	3.48 ± 0.64
VP8	10.68 ± 2.35
VP9	4.43 ± 1.84
VP10	38.65 ± 3.43
VP11	1 ± 0.07

[a] Virus-infected cells were pulse-labeled for 15 min and fractionated on polyacrylamide gradient gels, and the level of each polypeptide was calculated (see Materials and Methods). The results are expressed relative to the amount of VP11, which was given an arbitrary value of 1.

five genes, 1, 5, 8, 9, and 11. Finally, the lowest levels of mRNA accumulation (14,000 to 21,000 molecules per cell) were shown by three genes, 4, 6, and 10.

Quantitation of viral polypeptide production. It was possible that the different levels of mRNA detected in the experiments described above would be reflected directly in the amounts of the corresponding viral proteins, indicating that control of viral gene expression was primarily transcriptional. Since the complete RNA protein-coding assignments for the UKtc bovine virus used in this study are known (14), it is feasible to quantitate the synthesis levels of the various virus-encoded proteins and see whether their amounts do indeed directly correspond to those of their respective mRNAs.

The production of viral proteins during a 15-min pulse with [^{35}S]methionine was measured at 6.5 h postinfection. After labeling, the infected cells were fractionated on 5 to 11% polyacrylamide gradient gels, which were then dried and autoradiographed. Individual viral protein bands were excised from the gel, and the ^{35}S that they contained was counted. The results obtained were adjusted for the background radioactivity, the methionine content of the protein (when known), and the approximate molecular weight of the final protein product (13) and expressed relative to VP11, which was assigned a value of unity (Table 1). Data from five independent experiments were combined, and their mean is presented in Table 1. Of the 11 final gene products, 9 could be resolved completely; it was difficult to achieve the complete resolution of VP3 and VP4, and so the data for these were combined (Table 1).

From Table 1 it is clear that not all of the viral proteins were being synthesized in equal amounts at 6.5 h postinfection. Thus, VP6 and VP10 were both produced in large excess over the remaining nine proteins. VP6 is the major component of the inner capsid shell of the virus and carries the group antigen determinants of group A rotaviruses (23, 29), and VP10 is a nonstructural glycoprotein (4). VP8, which has not yet been unequivocably identified as being structural or nonstructural, was also synthesized in relatively large quantities, although only to about half that of VP6. The apparent predominance of VP8 in protein profiles of infected cells (Fig. 3) was due in part to its high methionine content (4.7%) relative to other viral proteins. VP9, the second clearly established nonstructural protein, and the VP7 glycoprotein complex, which makes up the major neutralization antigen found in the outer shell of the virion, were both produced in an approximately fourfold excess

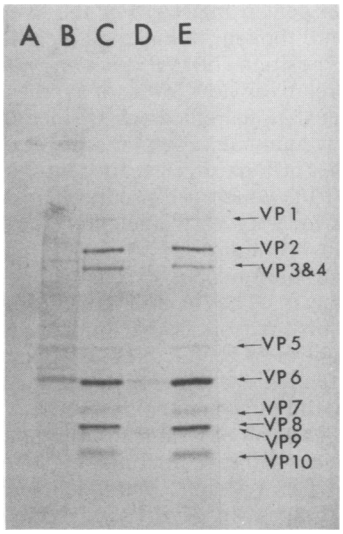

FIG. 3. Selective transcription in the absence of host cell protein synthesis. Infected cells treated with cycloheximide were pulse-labeled for 15 min at 6.5 h postinfection as described in Materials and Methods. Samples were analyzed on 5 to 11% polyacrylamide gradient gels. Lanes: A, cycloheximide-treated uninfected cells; B, untreated uninfected cells; C, untreated infected cells at 6.5 h postinfection; D, cycloheximide-treated infected cells labeled 15 min after removal; E, cycloheximide-treated infected cells labeled 3 h after removal.

over VP11. The remaining proteins are all structural components of the virion, and each was produced in very small amounts relative to VP11. For example, VP1 was synthesized at only 1/260 of the rate of VP10 at this time postinfection.

Having determined the relative levels of synthesis of both individual viral mRNAs and proteins, we could compare the two. When this was done (Table 2), it became clear that the level of mRNA for a particular gene was not directly reflected in the relative amount of the corresponding protein. For example, gene 10 mRNA accumulates to about one-third of that for gene 7 at 6.5 h postinfection, whereas VP10 is being synthesized at about four times the level of VP8. This suggests that gene 10 mRNA is being translated at 12 times

TABLE 2. Analysis of levels of transcription and translation for the 11 rotavirus genes[a]

Protein/gene	Production of proteins[b]	Accumulation of mRNA[b]	Translation-transcription frequency
VP1/SP1	0.15	1.3	0.1
VP2/SP2	1.32	2.3	0.5
VP3/SP3	1.27	1.3	0.6
VP4/SP4		0.6	
VP5/SP5	1.57	0.9	0.6
VP6/SP6	25.02	0.7	36.6
VP7/SP8	3.48	0.9	3.9
VP8/SP7	10.68	2.8	3.8
VP9/SP9	4.43	0.9	4.9
VP10/SP10	38.65	0.7	54.3
VP11/SP11	1	1	1

[a] The results presented in this table were taken from Fig. 2 for the transcriptional data and Table 1 for the translational data.
[b] Relative to VP11.

the efficiency of gene 7 mRNA. For the five proteins other than VP6 that are thought to make up the inner capsid shell (although the position of VP5 has not been precisely defined), their relatively low levels of synthesis appear to be due to poor translational efficiencies, since the mRNAs for these proteins accumulate to similar or higher quantities than some of the other mRNA species. By contrast, proteins such as VP6 and VP10, which are produced in large amounts, have mRNAs which are very efficiently translated, whereas VP8 appears to rely more on producing high levels of an inefficiently translated mRNA to achieve its relatively high levels of synthesis. The overall conclusion that can be reached from this analysis is that both transcriptional and translational control of gene expression operate in the rotavirus system to achieve the regulated synthesis of viral proteins during the viral replication cycle.

Qualitative regulation of viral transcription. Lau et al. (12) used the protein synthesis inhibitor cycloheximide in the mammalian reovirus system to obtain evidence for an early-late switch in the pattern of viral gene transcription. With appropriate adjustments for the differing kinetics of virus replication, a similar strategy was applied to the rotavirus system. Cycloheximide (20 μg/ml) was applied to the cells 2 h prior to infection and maintained until 6.5 h postinfection. The cycloheximide was then removed, and the cells were pulse-labeled for 15 min with [^{35}S]methionine at 15 min and at 3 h after removal. The labeled samples were balanced with respect to acid-precipitable radioactivity, and the proteins present were fractionated on gradient polyacrylamide gels. After autoradiography the protein profiles were examined with a view to identifying any viral protein being synthesized 15 min after the removal of the protein synthesis inhibitor. The results (Fig. 3) showed that as early as 15 min after removal of the block, four virally encoded proteins, VP5, VP6, VP8, and VP9, could be detected. On the basis of relative band intensities, both VP5 and VP9 appeared to be being synthesized at near-normal levels, whereas VP6 and VP8 had not completely recovered their normal levels of synthesis so early after release of the protein synthesis block. The production of these four proteins so soon after the removal of the cycloheximide implied that the transcription of the genes encoding them (genes 5, 6, 7, and 9, respectively) was independent of protein synthesis in the infected cells. The quantities of viral mRNA for these genes in cycloheximide-treated cells should therefore reflect the pattern of protein synthesis shortly after the block was released. RNA was extracted from cycloheximide-treated cells at 6.5 h postinfection, and the levels of mRNA accumulated for genes 5, 6, 7, and 9 were measured. The level of transcription of these genes was then compared with the level of transcription of genes 3 and 10, whose protein products, VP3 and VP10, respectively, were both absent from the protein profile made 15 min after removal of cycloheximide (Fig. 3). The pattern of protein synthesis seen immediately after removal of the protein synthesis inhibitor did indeed reflect the levels of mRNA present in the infected cells (Table 3). Although all the viral genes examined showed a reduction in their levels of accumulation in the presence of cycloheximide, the drop seen for the genes that were expressed shortly after removal of the block was much lower than for those whose protein products could not be detected. Thus, accumulation of mRNA for genes 5, 6, 7, and 9 was reduced by between 1.5- and 6.5-fold, whereas for genes 3 and 10 there was a 34- and 13-fold reduction, respectively.

Kinetics of minus-sense RNA synthesis. The results obtained for the accumulation of the minus-sense RNA strands of each viral gene are shown in Fig. 4. Since extraction of genomic dsRNA from purified virions shows that each of the 11 genes is present in equimolar amounts, it was expected that the 11 minus-sense RNAs would accumulate to the same level, but this clearly did not occur. There was as much as an 8.5-fold excess of minus-sense RNA for genes 1, 4, and 9 over that for gene 10, and since the variability of the assay being used did not exceed 20% (9), this difference appears to be a real one. The assumption that the strategy of rotavirus replication will follow the nonconservative mechanism established for mammalian reoviruses (1) precludes the possibility that the excess minus-strand RNA apparently made for some genes was free in the cell cytoplasm, and so the possibility is suggested that defective virus particles, lacking some of the viral genome segments, were being generated.

TABLE 3. mRNA accumulation in the presence of cycloheximide

Gene	Molecules of RNA accumulated/cell		
	Untreated cells	Treated cells	Fold reduction
SP5	20,470	15,300	1.3
SP6	15,795	2,870	5.5
SP7	63,900	12,500	5.1
SP9	21,180	3,290	6.4
SP3	28,400	835	34.0
SP10	16,960	1,300	13.0

[a] The accumulation of plus-sense transcripts (mRNA) for six of the viral genes in virus-infected cells maintained in the presence and absence of cycloheximide was measured as described in Materials and Methods.

On the basis of the temporal patterns of synthesis of minus-sense RNA, the viral genes could be divided into four groups. In the first group, which consisted of genes 2 and 9, minus-sense RNA could already be detected in the earliest sample taken (1.5 h postinfection). Minus-sense RNA of the sole member of the second group, gene 1, began to accumulate at 3.5 h postinfection. The third group consisted of four member genes, 6, 3 and 4, and 11, whose accumulation of minus-sense RNA was first detected at 4.5, 6.5, and 7.5 h postinfection, respectively. The accumulation of minus-sense RNA for the final group of four genes (5, 7, 8, and 10) was slow compared with the other groups and reached the lowest final levels.

By analogy with the replication strategy established for reovirus (1), it had been predicted that minus-sense RNA would not begin to accumulate until the later phases of the growth cycle (production of progeny virions was first detected at 3.5 h postinfection; Fig. 1). For eight of the viral genes, this appeared to be true, with accumulation either accelerating after this time (genes 3, 4, 6, and 11) or increasing slowly and steadily during the later stages of the growth cycle (genes 5, 7, 8, and 10). However, for genes 2 and 9, and to a lesser extent for gene 1, minus-sense RNA accumulated rapidly earlier during the infection cycle prior to progeny virus production. The possible significance of this rather unexpected temporal pattern of minus-strand synthesis is at present unclear.

DISCUSSION

The strand-specific probes used in the solution hybridization method developed for this study have allowed accurate quantitation of the pattern of synthesis of both plus- (mRNA)

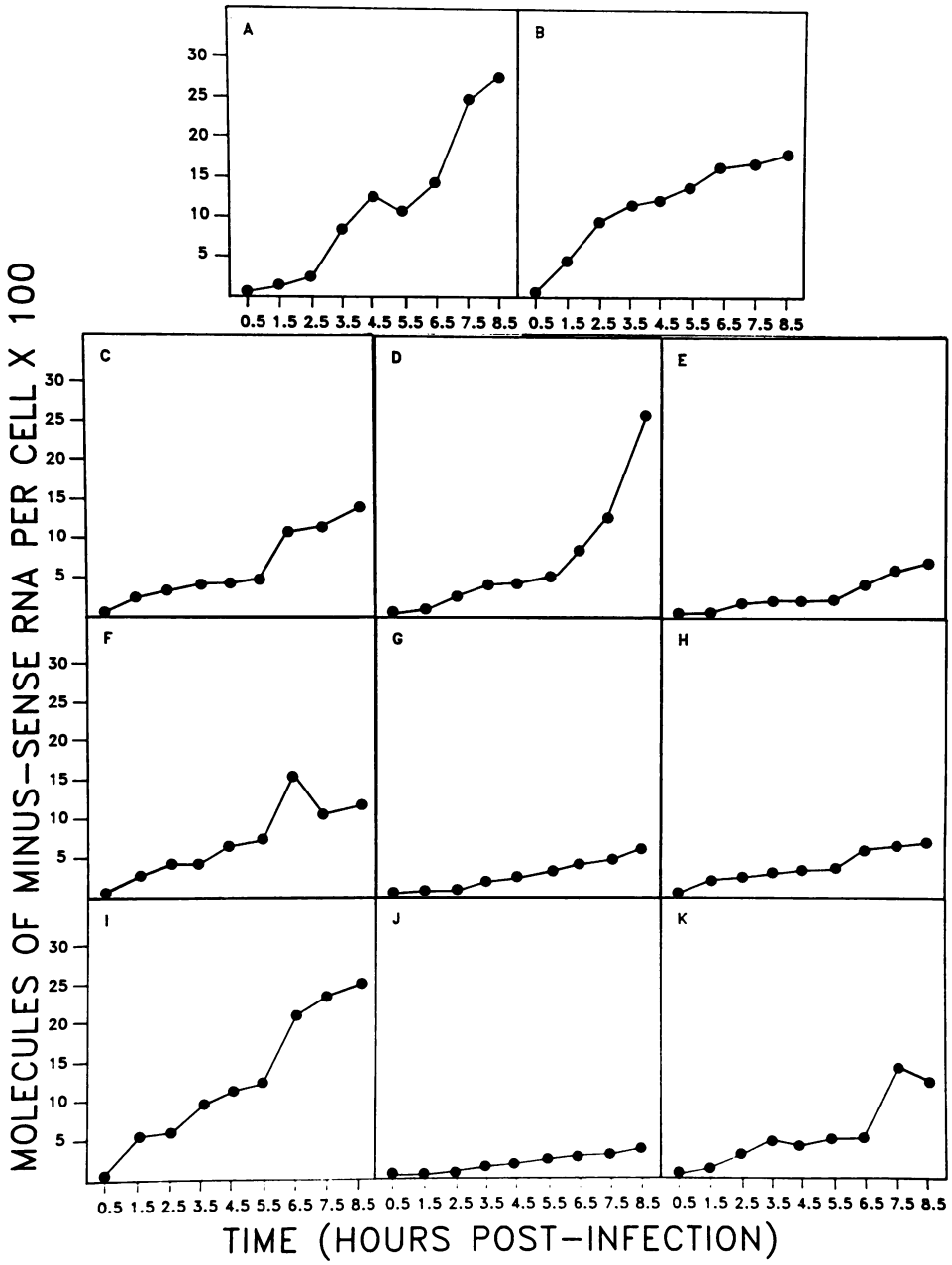

FIG. 4. Accumulation of minus-sense RNA. The figure shows the accumulation of minus-sense RNA strands occurring in a single-step growth curve carried out as described in Materials and Methods. The results obtained for genes 1 to 11 expressed in terms of molecules of RNA per cell are shown in panels A to K, respectively.

and minus-sense RNA strands for each of the 11 segments of rotavirus RNA. In this respect, it has been able to provide much more detailed information than could be obtained by the previous generation of assay used for this type of analysis of dsRNA viruses (20). It has the additional advantage that it allows the actual numbers of RNA molecules synthesized per cell to be measured rather than relying only on relative values of one species to another.

Examining the pattern of transcription for the different genes does allow some speculation of the function of some of the gene products; for example, VP8, the product of gene 7, is synthesized in large amounts very early in the replication cycle. The location of VP8 is still equivocal; in some viruses (SA11 [6]) it is categorized as being nonstructural, whereas in the UKtc strain it appears to be a structural protein (13). Irrespective of its location, this protein has been shown to have RNA-binding properties (H. N. Baybutt and M. A. McCrae, unpublished observation). This, together with its early accumulation, suggests that it may play a role in the early stages of morphogenesis, such as the assembly of sets of mRNA into immature virions.

In earlier studies (13) we acknowledged that temporal control of gene expression was probably operating in this virus system, since three temporal classes of polypeptide could be defined. The first, which included VP9, reached the maximum rate of synthesis very early (2 h postinfection) in

the replication cycle. In the second group were proteins such as VP1, whose maximum rate of synthesis was not seen until later in the cycle (4 h postinfection). Finally, there were proteins such as VP6, whose rate of synthesis appeared to increase steadily throughout the infectious cycle. The pattern of plus-strand RNA synthesis (Fig. 1) shows, however, that for all of the genes, the levels of mRNA continue to increase until at least 7.5 h postinfection. Some form of general translational control must therefore be operative, otherwise the rate of synthesis of all the viral proteins would be expected to increase throughout the cycle; i.e., the frequency of translation for mRNAs of the first two groups of proteins must decrease with time. It is possible that there is some form of structural modification of these mRNAs which, although it affects their translatability, does not prevent them from hybridizing to the RNA probes used in this study. The inability of the current assay to distinguish between functional and nonfunctional mRNA molecules obviously has to be taken into account when trying to interpret the accumulation data.

Despite the temporal control of transcription that was seen for the various genes, under normal infection conditions it was not possible to see any clear qualitative switching of transcription of the early-late type of transition observed for a number of DNA viruses such as simian virus 40 and vaccinia virus (8, 21, 22). In their studies of reovirus, Lau et al. (12) were able to use the protein synthesis inhibitor cycloheximide to define a group of so-called early genes whose transcription was independent of protein synthesis in the infected cell, and these authors speculated that the protein products of these genes may play a regulatory role in the replication cycle. By analogy, the similar experiment carried out in this study revealed a group of four genes (genes 5, 6, 7, and 9) whose transcription was similarly partially independent of host protein synthesis. It is possible that the protein products of these genes, VP5, VP6, VP8, and VP9, respectively, also have some regulatory functions in the viral growth cycle, and certainly the RNA-binding properties of VP8 would befit it for such a role. Further studies are clearly needed to substantiate this putative regulatory role for these early genes.

The levels of minus-strand accumulation during the replication cycle were in all cases much lower than those of plus-strand accumulation, ranging from 6- to 130-fold lower. This suggests that the majority (83 to ≥99%) of the plus-sense transcripts function solely as templates for protein synthesis, with the minority serving as the template for the addition of minus-sense RNA and hence progeny genome production. By analogy with the reovirus system, the presence of equimolar amounts of the 11 RNAs in infectious rotavirus particles suggested that there should be equimolar quantities of the various minus strands synthesized in infected cells. When the quantities of minus strands accumulated were compared with the yield of infectious virus particles and the particle-to-PFU ratio, the percentage of the minus-sense RNA that could be accounted for as being present in virus particles varied for the different RNA segments. Thus, for gene 10, which accumulated to the lowest levels, 300 molecules per cell had accumulated by 8.5 h postinfection. This compares with the yield of infectious virus at this time of 54 PFU per cell, which, based on the measured particle-to-PFU ratio of 4.7:1, represents 254 molecules of gene 10 minus-sense RNA in recognizable virions, i.e., 84% of the total. At the other extreme was gene 1, with only 9.8% of the minus-strand RNA accumulated being accounted for in identifiable virus particles. The conclusion of such an analysis was that for some of the RNA segments, the amount of minus-strand RNA accumulated corresponded well to the number of virus particles produced, whereas in other cases, most notably genes 1, 4, and 9, there was a vast overproduction of such RNA. For genes giving the highest levels of minus-strand accumulation, only about 2% of the RNA became part of a virion capable of initiating infection, suggesting that the replication process is extremely inefficient. Since these experiments were carried out with cells infected at a high input multiplicity, it is feasible that large numbers of defective particles may have been generated which contained complete copies of genes 1, 4, and 9 but which lacked one or more of the other genome segments and hence were sufficiently unstable that they did not survive to be counted as recognizable virions in the electron microscope. Defective virus particles deleted for a number of genomic RNA segments have previously been reported for both influenza virus (16, 17, 24) and reovirus (2, 19, 26). However, to date, similar defective particles have not been reported for the rotavirus system, and so further work is needed to substantiate the production of defective virions as being the reason for the large disparities in the levels of accumulation of the different minus-sense RNAs seen in this study.

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