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Biochemistry

Sequential transcription of the genes of vesicular stomatitis virus

(ultraviolet irradiation/target sizes/gene order)

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ABSTRACT Increasing exposure of vesicular stomatitis virus particles to ultraviolet radiation caused differential inhibition of the synthesis *in vitro* of individual mRNA species which code for the viral structural proteins L, G, M, NS, and N. The synthesis of each mRNA species showed single hit kinetics. Target sizes for the expression of each gene were derived from kinetic data, and the molecular weight of the mRNA coding for the N protein was found to be proportional to the target size of its corresponding gene. The remaining genes had target sizes larger than expected, ranging up to the molecular weight of the entire genome of the vesicular stomatitis virus. These results suggest that RNA transcription is initiated at a single site on the RNA genome and that individual mRNA species are synthesized sequentially. This analysis has allowed the mapping of the genes of this virus in the order 5′-L-G-(M-NS)-N-3′.

Vesicular stomatitis virus (VSV) contains a single-stranded RNA genome of negative polarity with a molecular weight of approximately 4×10^6 (1). A virion-associated RNA polymerase transcribes the genome RNA *in vitro* or *in vivo* into monocistronic mRNAs which can be translated into viral polypeptides in protein-synthesizing systems *in vitro* (2). mRNA species of molecular weights 2.1, 0.7, and 0.55×10^6 code for the large protein (L) (3), the surface glycoprotein (G), and the nucleocapsid protein (N) of the virion, respectively. Two mRNA species of similar molecular weights (0.33×10^6) code for the remaining membrane (M) and minor (NS) proteins (2). Together, these five mRNAs account for almost all of the coding potential of the VSV genome (4).

The precise mechanism for the synthesis of VSV mRNAs is not known. The five monocistronic mRNAs may be synthesized by initiation at multiple sites on the genome RNA, or alternatively, the RNA polymerase may initiate transcription at a single site with subsequent cleavage of the mRNAs from a precursor RNA molecule. The latter model, involving RNA processing, gains indirect support from the following experimental observations. (*a*) The 5′-termini of the VSV mRNA species synthesized *in vitro* are blocked, having the structure GpppAp . . . (5). Since only the α phosphate of ATP and both the α and β phosphates of GTP are incorporated into the blocked structure, the biosynthesis of this structure may involve the cleavage of a larger RNA molecule generating a free 5′-phosphate which is then blocked with GDP. (*b*) The 5′-terminal sequence of all of the VSV mRNA species is the same: GpppApApCpApGp . . . (6) and the 3′-terminal sequence of the VSV genome is . . . $PypGpU_{OH}$ (7). Since these two sequences are not complementary, it follows that synthesis of the mRNAs must commence distal to the 3′-terminus of the genome RNA. Furthermore, to synthesize the complementary 42 S + strand (a required intermediate in the replication process), the RNA polymerase must initiate transcription at the 3′-terminus. These observations together suggest that both types of RNA may be initiated at the same site and that mRNA production involves subsequent processing while the 42 S + strand is not cleaved. (*c*) The recent demonstration that a discrete RNA molecule 60–70 nucleotides long with a 5′-terminal sequence ppApCpGp . . . complementary to the 3′-terminal sequence of VSV genome RNA is released during transcription *in vitro* is consistent with a model involving RNA processing *in vitro* (16).

In this communication we have used ultraviolet (UV) irradiation to produce pyrimidine dimers in the VSV genome RNA (8) and so prevent transcription beyond the damaged site (9). If the mRNAs are synthesized by independent initiations, the target-sizes of the genes will be proportional to the molecular weights of the corresponding mRNA species. On the other hand, if the mRNAs are processed from a precursor RNA molecule having a single site for initiation, the synthesis of a particular gene will depend on the prior synthesis of its 3′-proximal genes. Accordingly, the target-size of that gene will include the sum of the molecular weights of its 3′-proximal genes. If the latter mechanism is true, the gene order of VSV can be established.

MATERIALS AND METHODS

UV Irradiation of VSV. VSV (Indiana serotype) was grown and purified as described previously (10). Virus preparations (25 μl) were disrupted in the presence of Triton and 0.4 M NaCl (11), diluted to 0.1 ml with water, and placed in cylindrical plastic caps 6 mm in diameter to give an average depth of solution of approximately 3 mm. Such preparations were exposed to an incident UV irradiation of 1 erg sec^{-1} mm^{-2} supplied by a 253.7 nm wavelength source situated 30 cm above the samples. Exposures were done at 4° with gentle agitation to ensure uniform irradiation.

RNA Transcription *In Vitro*. Disrupted virus preparations from above were used to prime standard 0.2-ml transcriptase assay mixtures (11) using 10 μCi of [^{3}H]UTP as the labeled precursor. After incubation, aliquots of purified 14.5S VSV mRNA (2) labeled with [α-^{32}P]UMP were added to each reaction, and the mixtures were layered on cushions of 20% (wt/wt) glycerol in 0.8-ml adaptor tubes for the Beckman SW50.1 rotor. After centrifugation for 1 hr at 46,000 rpm to sediment the nucleocapsids and transcriptive intermediates, the supernatant product RNAs were recovered and processed as described in the figure legends. The addition of the ^{32}P-labeled internal standard to each sample allowed a standardization for losses incurred during handling and processing.

RESULTS

Effect of UV Irradiation on VSV mRNA Synthesis *In Vitro*. Pyrimidine dimers produced by UV irradiation of RNA will cause the termination of RNA transcription at the sites where the dimers occur (9). Undamaged genes should be transcribed with proper initiation, elongation, and termination of the RNA chains. In order to demonstrate these effects, we

Abbreviations: VSV, vesicular stomatitis virus; UV, ultraviolet.

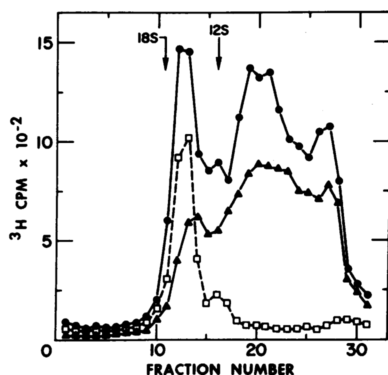

FIG. 1. Velocity sedimentation analysis of RNA products synthesized *in vitro* by VSV particles after UV irradiation. A VSV preparation was disrupted with Triton X-100 and 0.4 M NaCl and irradiated for 60 sec as described in *Materials and Methods*. The preparation was then diluted in a standard transcriptase assay (11) containing [^{3}H]UTP and incubated for 3 hr. The resulting labeled RNA products were extracted with phenol and precipitated with ethanol, and one portion was analyzed directly by sedimentation through a 15–30% (wt/vol) sodium dodecyl sulfate sucrose gradient (4) for 17 hr at 33,000 rpm in a Beckman SW40 rotor. The remaining portion was fractionated by oligo(dT)-cellulose chromatography into unbound RNA and poly(A)-containing RNA as described previously (12). Portions of each preparation were analyzed separately by sedimentation as described above. All three gradients were fractionated and assayed for radioactivity as described previously (12). (●) Total RNA product; (□) poly(A)-containing RNA; (▲) RNA not bound to oligo(dT)-cellulose.

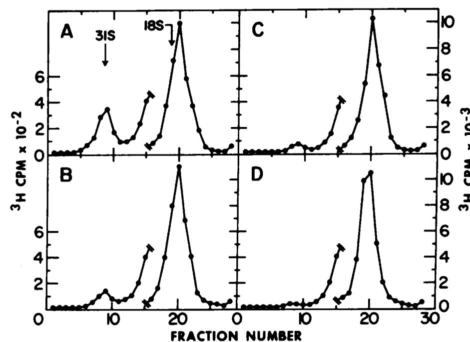

FIG. 2. Velocity sedimentation analysis of the poly(A)-containing RNA synthesized *in vitro* by VSV preparations after varying doses of UV irradiation. Aliquots of a disrupted VSV preparation were exposed for varying times to UV radiation as described in *Materials and Methods*. Portions of the poly(A)-containing, [^{3}H]UMP-labeled RNA synthesized by each sample (see Fig. 1) were analyzed by sedimentation as described in the legend for Fig. 1 except that centrifugation was at 24,000 rpm. Approximately equal amounts of radioactivity were layered on each gradient. Note that the breaks in the curves indicate a 10-fold change in the scale. (A), (B), (C), and (D) represent the sedimentation profiles of transcribed RNA after exposure of VSV to UV irradiation for 0, 5, 10, and 15 sec, respectively.

exposed Triton-disrupted VSV particles to a known dose of UV irradiation and used the resultant preparation to prime a standard RNA transcriptase reaction containing [^{3}H]UTP as the labeled precursor (11). A portion of the labeled RNA product was analyzed directly for low-molecular-weight RNA species by velocity sedimentation (Fig. 1), and the remainder was subjected to oligo(dT)-cellulose column chromatography to select for the poly(A)-containing RNA. Because the poly(A) is located at the 3′-termini of the *in vitro* mRNAs, the oligo(dT)-bound fraction represents only those mRNA molecules that are completed (10). The unbound RNA (eluting at high salt) and bound RNA (eluting at low salt), were isolated and analyzed separately by velocity sedimentation (Fig. 1). Control VSV preparations without irradiation transcribe mRNA species that sediment characteristically at 17 S, 14.5 S, and 12 S under these centrifugation conditions (see Fig. 3A), and have been shown previously to code for the G, N, and both the M and NS proteins, respectively (2). The UV-treated RNA synthesized by VSV contained a large amount of heterogeneous RNA (Fig. 1) smaller than 12 S that presumably represented prematurely terminated RNA chains. After oligo(dT)-cellulose fractionation, the poly(A)-containing RNA products sedimented as unique RNA species, whereas the unbound RNA products sedimented heterogeneously as smaller RNA molecules. These results demonstrated that this dose of UV irradiation caused the majority of the product RNAs to be small and prematurely terminated since they lacked poly(A) sequences (see Fig. 3A). The RNA products that were polyadenylylated were assumed to reflect the complete transcription products of those genes that escaped UV damage. Thus, by this procedure it was possible to separate the complete mRNA molecules from the prematurely terminated ones and to establish a value of n/n_0 for a particular mRNA species, where n = the amount of the intact mRNA species synthesized after a given UV dose and n_0 = the total amount of the corresponding mRNA species synthesized in the absence of irradiation (see below). Using the oligo(dT)-cellulose fractionation procedure described above, we determined the total poly(A)-containing RNA synthesized by VSV irradiated with UV for different time periods. The percentages of control values were plotted against time on a semilogarithmic scale (data not shown). The RNA synthesis decreased linearly, with only 14% of the original RNA being synthesized after UV exposure for 60 sec, indicating the profound inhibitory effect irradiation had on RNA transcription.

To show that the RNA genome and not the polymerase was being inactivated by the UV irradiation, we did a complementation experiment. Two VSV preparations, one of which was irradiated, were disrupted and the ribonucleoprotein cores and the soluble transcriptases were separated (11). After cross reconstitution assays, it was found that the UV-irradiated cores were inactive in RNA transcription, whereas the soluble transcriptases from the same preparation were fully active (data not shown).

In separate experiments, effects similar to those caused by UV irradiation were obtained when the genome RNA was partially hydrolyzed by exposure of high salt VSV ribonucleoprotein cores (11) to mild alkaline conditions (pH 10.5) prior to RNA transcription (data not shown).

Kinetics of Synthesis of Individual VSV mRNA Species after UV Irradiation. In order to study the rate of decrease of individual VSV mRNAs after UV irradiation, aliquots of purified VSV were first subjected to UV irradiation for various time intervals, and the transcribed poly(A)-containing mRNAs were purified and analyzed by velocity sedimentation. As shown in Fig. 2, the synthesis of the VSV 31S RNA species (referred to as L-message) progressively decreased to only 4% of the control value after 15 sec of UV exposure. Due to the large size of the 31S RNA (molecular weight 2.1 × 10^6), low-speed centrifugation was used, and consequently the smaller mRNA species sedimented together at 12–18 S in these gradients. To analyze the latter mRNA species, we centrifuged aliquots of the poly(A)-containing RNAs synthesized after increasing doses of UV at higher speed to achieve better resolution of the component species. For quantitation of the amount of each mRNA species accurately from the gradient profiles, purified 14.5S RNA species synthesized *in vitro* by unirradiated VSV in the presence of [α-^{32}P]UTP was added to each RNA product as an internal standard. The rate of decrease of each mRNA species

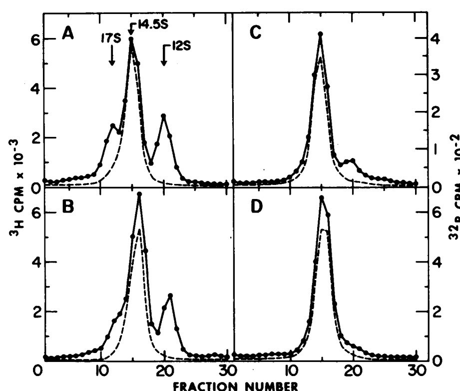

FIG. 3. Velocity sedimentation analysis of the poly(A)-containing RNA synthesized *in vitro* by VSV preparations after varying doses of UV irradiation. Portions of the [^{3}H]UMP-labeled RNA samples (●—●) described in the legend for Fig. 2 were analyzed by sedimentation as described in the legend for Fig. 1. Included in each gradient (- - - -) is a sample of purified 14.5S VSV mRNA labeled with [α-^{32}P]UMP as an internal standard (see *Materials and Methods*). (A), (B), (C), and (D) represent the high-speed sedimentation profiles of transcribed RNA after exposure of VSV to UV irradiation for 0, 10, 40, and 110 sec respectively.

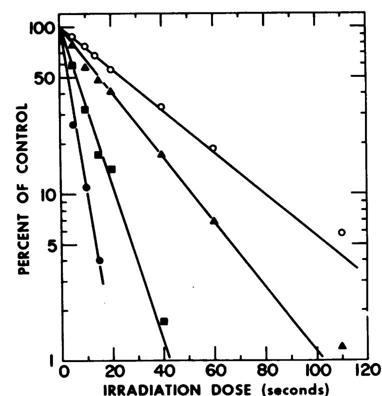

FIG. 4. The effect of increasing UV irradiation of VSV on the synthesis of poly(A)-containing mRNA species. From a series of profiles similar to those seen in Figs. 2 and 3, the proportional synthesis of each mRNA species was determined for varying doses of UV irradiation. The presence of the purified N-message as an internal standard in each gradient (Fig. 3) enabled the precise quantitation of each mRNA species. The synthesis of individual mRNAs at each UV dose is expressed as a percentage of the synthesis of the corresponding mRNA transcribed from a VSV preparation without prior irradiation. (●) Synthesis of the L-message; (■) synthesis of the G-message; (▲) synthesis of the (NS + M)-messages; (O) synthesis of the N-message.

at representative UV irradiation doses is shown in Fig. 3, and a differential effect of UV on the rate of synthesis of individual mRNA species can be seen. After irradiation for 40 sec, the synthesis of the 17S RNA (G-message) was virtually abolished, whereas the synthesis of the 12S RNA (M and NS messages) was still detectable but was eliminated after exposure for 110 sec. On the other hand, the synthesis of the 14.5S RNA (N-message) was highly resistant to UV, and considerable synthesis was still detectable even after exposure for 110 sec. Fig. 4 summarizes the rate of decrease of RNA synthesis of each message after various times of UV irradiation. The following points can be made from these results: (*a*) the rate of decrease of mRNA synthesis for each gene was linear, indicating single-hit kinetics; (*b*) the cessation of synthesis of the messages was sequential and in the order L, G, (M + NS), and N; and (*c*) although the physical size of the N gene is larger than that of the M or NS genes, transcription of the former was more resistant to UV and was the last to be eliminated. The observations suggest that the N-gene is located close to a single initiation site for transcription and that the transcription of the remaining genes is sequential with the L-gene being located furthest from the initiation site.

Determination of the Target Sizes of the Individual VSV Genes. The inhibition of synthesis of each mRNA follows single-hit kinetics (Fig. 4) and so, according to target theory, the effect of UV irradiation on the synthesis of each mRNA species *in vitro* can be expressed as $\ln (n/n_0) = -K$ (dose), where n/n_0 = the amount of mRNA synthesized at a given UV dose relative to no irradiation. Since K is an inactivation constant which is proportional to the target-size of the corresponding gene (13), the equation can also be expressed as $\ln (n/n_0) = -C{\cdot}T{\cdot}D$, where C = a constant, T = the target size of the gene, and D = the UV dose or time of exposure (13). Since the transcription of the N-gene is most resistant to UV damage (Figs. 3 and 4), it was concluded that this gene must lie closest to the initiation site for transcription (i.e., near the 3′-terminus of the VSV genome RNA). Therefore, the value of 0.55×10^6 may be substituted for T, assuming that the molecular weight of the N-message corresponds to the target size for the N-gene. At $n/n_0 = 1/e = 37\%$ resistance for the N-gene after 34 sec of UV exposure, the value of C was calculated to be -5.317×10^{-8} from the above equation. This value for C was then re-substituted in the equation to calculate the target sizes of the other VSV mRNAs at 37% resistance. The results in Table 1 show that the values obtained for the calculated target-sizes for each gene did not correlate with the actual molecular weights of their transcribed mRNAs. In fact, the target size for a particular gene appears to include the sum of the molecular weights of its 3′-proximal genes. For example, the calculated target-size of the L-gene (molecular weight 4.06×10^6) is nearly equal to the sum of the molecular weights of all five messages (4.01×10^6). The latter value is also the approximate molecular weight of the VSV genome RNA. This suggests that a single UV hit anywhere on the genome will prevent the synthesis of completed L-message, and the synthesis of the L-message *in vitro* is dependent on the prior synthesis of all of the other messages. Similarly, from Table 1, it can be concluded that the synthesis of the G-message is dependent on the prior synthesis of the N and (NS + M) messages, and that the syntheses of the (NS + M) messages are dependent on the synthesis of N-message. An interpretation of the results is that the virion-associated RNA polymerase of VSV initiates transcription at a single site with the N-gene located closest to the 3′-terminus and the L-gene closest to the 5′-terminus. Since analytical methods are not available for the resolution of the NS and M-messages due to their very similar sizes, the effect of UV irradiation on the synthesis of both mRNAs was studied as that on a single transcriptional unit. Consequently, the observed target sizes for both messages were equal (Table 1), and the estimated target sizes were derived by adding the molecular weight of either to that of the N-message.

It should be noted that the constant C was determined assuming the target-size of the N-gene to be equal to its molecular weight. When the values of C were determined using the molecular weights of the (M or NS), G, or L messages, the target-sizes of the remaining genes were quite different from the values shown in Table 1. For example, the target-size of the N-gene was calculated to be 0.2×10^6, 0.18×10^6, and 0.28×10^6, using the molecular weights of the (M or NS), G, or L-messages, respectively. These values are two to three times less than the actual molecular weight of the N-message. Thus, for the five messages synthesized *in vitro*, only the target-size of

Table 1. Comparison of the target sizes and the molecular weights of the VSV mRNA species

Genes	Molecular weight of mRNA ($\times 10^{-6}$)	Observed target sizes* ($\times 10^{-6}$)	Estimated target sizes† ($\times 10^{-6}$)
N	0.55	0.55	0.55 (N)
NS	0.33	0.85	0.88 (N + NS)
M	0.33	0.85	0.88 (N + M)
G	0.70	2.06	1.91 (N + NS + M + G)
L	2.10	4.06	4.01 (N + NS + M + G + L)

* Observed target sizes were calculated as described in the *text* using the data from Fig. 5. The times of UV irradiation resulting in a 37% residual expression of each gene were N = 34 sec, (NS + M) = 22 sec, G = 9 sec, and L = 4–5 sec.

† Estimated target sizes in each case were derived by the inclusion of the molecular weights of all 3′-adjacent genes as indicated by the parentheses.

N-gene agrees with the physical size of the corresponding message. On the other hand, the target-sizes of the remaining genes are larger than their corresponding physical sizes, depending upon their location in the genome RNA.

Gene Order of VSV. From the results in Table 1, a transcriptional map of VSV can be constructed. Since the synthesis of the N-message is the most resistant to the action of UV, its location on the VSV genome RNA must be closest to the initiation site for transcription, and nearest to the 3′-terminus of the template RNA. From the calculations of the target sizes, the genes adjacent to the N-gene are the (NS + M) genes (whose messages cannot be separated by this analysis), followed by the G and then the L genes. Thus, VSV mRNA synthesis *in vitro* appears to involve a single initiation site for transcription at the 3′-terminus with the sequential transcription of the genes which are arranged in the order: 3′-N-(NS-M)-G-L-5′.

DISCUSSION

The negative-stranded genome RNA of VSV is transcribed *in vitro* by the virion-associated RNA polymerase into five monocistronic mRNAs that code for the five principal structural proteins of VSV (2). As discussed in an earlier report (6), mRNA synthesis *in vitro* can proceed either by initiations at multiple sites on the genome RNA, or by initiation at a single site followed by processing of the transcribed RNA molecule into cistron-sized mRNA species. Exposure of the VSV genome to UV irradiation prior to transcription has allowed differentiation between these two possibilities. The rate of decrease in synthesis of each mRNA after increasing UV exposure followed single-hit kinetics (Fig. 4). However, there was a significant differential effect of UV on the synthesis of the N and the (M + NS) messages. Although the molecular weight of the N message (0.55×10^6) was larger than either the M or NS messages (0.33×10^6), the syntheses of the latter were more sensitive to UV, indicating that the target-sizes of these genes were not proportional to their physical sizes. This result also suggested that the N-gene was located on the genome RNA close to the initiation site for RNA transcription. By substituting the molecular weight of the N-message in the equation $\ln (n/n_0) = -C$ (molecular weight) (time of UV exposure), the target-sizes of the remaining genes were calculated (Table 1). The results showed that the calculated target-sizes for these genes were considerably higher than the molecular weights of the corresponding mRNAs. However, the actual target-size for each gene was found to include the molecular weights of all 3′-proximal genes, indicating that the transcription of each gene was dependent on the prior expression of all 3′-adjacent genes. From these results, the order of the genes on the VSV genome was established as 5′-L-G-(M-NS)-N-3′. The orientation of the M and NS genes could not be determined because their messages could not be separated. However, the results in Table 1 indicate that these genes are contiguous and are situated between G and N. Since the M and NS proteins can be clearly resolved by gel electrophoresis, the order of these two genes may be determined by examination of their translation products *in vitro* after appropriate UV irradiation.

The data indicate a compulsory order for the synthesis of VSV mRNAs, and some previous observations can now be explained in terms of a model involving a single initiation site for the RNA transcription followed presumably by processing. The extent of synthesis of individual mRNA species *in vitro* by unirradiated VSV [L < G < (NS + M) < N, see Figs. 2 and 3] falls in the same order as their sensitivities to UV irradiation. Furthermore, the 5′-terminal structure of the VSV mRNA species, GpppA . . . , is possibly formed after cleavage of a precursor RNA molecule to expose a free 5′-phosphate moiety that is subsequently blocked by GDP. We have also observed that the 3′-terminal sequence of the VSV genome RNA, . . . $PypGpU_{OH}$ (7), is not complementary to the 5′-terminal sequence of the mRNA species, which is GpppApApCpApGp . . . (6). If the latter sequence originates after processing of the precursor RNA molecule, an initiator sequence transcribed from the 3′-terminus of the VSV genome must be present as an *in vitro* reaction product. In fact, we have recently shown that a discrete RNA species 60–70 nucleotides long with a 5′-terminal sequence ppApCpGp . . . is synthesized *in vitro* during the transcription process (16). This observation supports the present conclusion for a single initiation site for VSV mRNA transcription. However, an interpretation of the data that cannot be excluded is that the transcriptase enzymes synthesize and complete each mRNA in turn before re-initiating transcription of the next.

This novel strategy for mRNA synthesis by VSV may be linked to the replicative process of the genome RNA. It is possible that the entire complement of the VSV genome (42 S + strand), a necessary intermediate in the replicative process, may be synthesized in conditions where the presumed processing event is inhibited. In the infected cell, a viral or a host function may mediate this inhibition, since no 42 S + strand has yet been detected in *in vitro* conditions. Thus, the synthesis of the 42 S + strand for replication and the transcription of mRNAs (for translation) may be effected by the same enzyme, and both functions may be interchangeable in the life cycle of VSV, the extent of each being regulated by a processing enzyme.

Finally, possible RNA processing in the VSV system may resemble the processing of ribosomal RNA in eukaryotic or prokaryotic cells (14) and also the processing of mRNA in the T7 phage system (15). Further studies with VSV transcription will help elucidate the precise mechanism of mRNA synthesis

and the nucleotide sequence recognized during possible processing.

We thank Dennis Rhodes for helpful discussions.

Note Added in Proof: While this work was in progress, we learned that Ball and White (17) have come to a similar conclusion concerning the order of transcription of the genes of VSV using a coupled *in vitro* transcription and translation system, primed by UV-irradiated VSV.

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