Site on the RNA of an Avian Sarcoma Virus at Which Primer Is Bound

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In vitro transcription of the avian tumor virus RNA by RNA-directed DNA polymerase is initiated on a unique cellular 4S RNA. Previous studies have shown that on the average there is one such RNA primer hydrogen bonded to each viral 35S RNA. The present study confirms that finding and demonstrates that, at least for the majority of 35S RNA molecules, the primer is bound at a site close to the 5'-terminus.

The genome of the avian RNA tumor virus is in a 70S RNA aggregate that contains 2 to 4 molecules of 35S RNA (12) plus 4S tRNA (8) and ribosomal 5S RNA (8). The 35S RNAs are probably identical (1, 2, 16). In vitro the transcription of the 70S RNA into DNA by the viral RNA-directed DNA polymerase is initiated on a unique 4S RNA species (9) that is probably identical to the cellular tryptophan tRNA (5, 17). On the average there is approximately one primer binding site per 35S RNA, and in naturally occurring viral RNA at least 70% of these sites are filled (J. M. Taylor, B. Cordell-Stewart, W. Rohde, H. M. Goodman, and J. M. Bishop, Virology, in press). The present communication describes experiments undertaken to determine the location of the site (or sites) on the 35S RNA at which primer is bound.

MATERIALS AND METHODS

Virus growth and purification. Secondary cultures of chicken cells (C/O, gs-, Heisdorf and Nelson Laboratories) were infected at low multiplicity with avian sarcoma virus. Either strain B77 clone 9 (obtained from R. Riis) or a nontransforming derivative, td-B77 (obtained from P. Vogt), was used. The cells were grown in a large roller bottle (1585 cm² of surface area) with 25 ml of Ham F-10 medium containing 4% calf serum, 10% tryptose phosphate broth, and 20 μCi of [¹⁴C]uridine (27.8 Ci/mmol, New England Nuclear) per ml. The radioactive virus was purified (4) from a single harvest (24 h), subsequently pelleted, and resuspended in 0.2 ml of 0.1 M NaCl-0.01 M Tris-hydrochloride (pH 7.4)-0.001 M EDTA.

Preparation of 70S complexes between template and tagged primer RNA. To the resuspended virus was added 0.8 ml of a reaction mixture containing: 0.1 M Tris-hydrochloride, pH 8.1; 0.01 M MgCl₂; 2% mercaptoethanol; 0.01% Triton X-100; 10⁻⁴ M dGTP; 10⁻⁴ M TTP; 0.86 x 10⁻⁴ M [α-³²P]pATP (115.1 Ci/mmol, New England Nuclear); and 100 μg of actinomycin D (Calbiochem) per ml. After incubation for 30 min at 37 °C the reaction was stopped by the addition of sodium dodecyl sulfate (0.5%) and self-digested Pronase (500 μg/ml) and incubated a further 15 min at 37 °C. The 70S RNA was isolated by sedimentation (9) and subsequently collected by ethanol precipitation.

Fractionation of partially denatured 70S complexes on oligo(dT)-cellulose. The isolated 70S complex was partially denatured by heating at 59 °C for 5 min in 0.2 ml of 0.01 M Tris-hydrochloride (pH 7.4)-0.01 M EDTA, and then applied to a 8-g column of oligo(dT)-cellulose (Collaborative Research) in 0.5 M NaCl-0.01 M Tris-hydrochloride, pH 7.4. The RNA which failed to bind to the column is designated "poly(A) deficient" and the remainder, which was subsequently eluted with 0.01 M Tris-hydrochloride, pH 7.4, is designated "poly(A) containing." For example, in the experiment described in Fig. 1, the input RNA contained 187,000 counts/min of [³²P]Juridine-labeled RNA and 10,600 counts/min of bound primer specifically labeled with [³²P]. Of these 37 and 16%, respectively, bound to the column. The two fractions were subsequently concentrated by ethanol precipitation.

Sedimentation analysis. The poly(A) containing and poly(A)-deficient fraction were separately analyzed by sedimentation into gradients of 15 to 30% sucrose containing 0.01 M Tris-hydrochloride, pH 7.4; 0.01 M EDTA; and 0.01% sodium dodecyl sulfate. Centrifugation was for 15 h at 28,000 rpm and 22 °C in the SW40 rotor of a Spinco L5-50. Aliquots of the gradient fractions were precipitated with 10% trichloroacetic acid in the presence of 80 μg of calf thymus DNA carrier, collected onto glass fiber filters, and counted in a Beckman scintillation spectrometer. The [³²P]-labeled L cell rRNA marker was a gift of Dawn Kelley.

Polyacrylamide gel analysis. RNA fractions were analyzed by electrophoresis into cylindrical gels (0.6 by 8 cm) of 2.1% acrylamide cross-linked with 0.28% (vol/vol) ethylene diamine. Bromophenol blue was added to the sample prior to electrophoresis. The buffer present both in the gel and the electrode
chambers was 0.04 M Tris-0.004 M sodium acetate-0.001 M EDTA, adjusted to pH 7.4 with acetic acid. After electrophoresis, the gel was removed, frozen, and fractionated with an apparatus consisting of stacked razor blades (Hoefer Scientific). The gel slices were placed in scintillation vials and solubilized by treatment for 30 min at room temperature with 0.4 ml of 20% (vol/vol) ammonia. After the addition of 10 ml of cocktail (40% ethylene glycol monoethyl ether, 60% toluene, 2.4% Liquifluor [New England Nuclear]), the samples were counted in a Beckman scintillation spectrometer.

It is important to note that RNA aggregation has been detected in polyacrylamide gel analysis of poly(A)-containing RNA and results particularly in significant accumulation of RNA at the origin. This can be avoided by heating the RNA (in 0.01 M EDTA-0.01 M Tris, pH 7.4) at 59 C for 5 min prior to electrophoresis.

RESULTS

An endogenous reaction of detergent-disrupted avian sarcoma virus (strain B77, clone 9) was performed in the presence of dGTP, dTTP, and [α-32P]dATP, but with dCTP omitted. As previously documented, this reaction specifically labels the 4S RNA primer in the 70S RNA complex with a short oligodeoxynucleotide (20). After this, the 70S RNA complex was isolated by virtue of its sedimentation velocity and heated for 5 min at 59 C in a low salt buffer. Again as previously documented, this heating does not effect the binding between the 35S RNA and that 4S RNA which functions as primer but dissociates the 70S complex into 35S subunits and removes all 5S RNA and non-primer 4S RNA (4, 6).

We have sought to locate the site on the partially denatured 35S RNA at which the specifically labeled primer is bound. To do this we have made use of the observations that the majority of viral 35S RNA contains a sequence of about 180 residues of adenyllic acid (13), and that this poly(A) is located at the 3’-terminus of the 35S RNA (19, 21). The rationale was to obtain fragments of partially denatured 35S RNA that contain an intact 3’-terminus of poly(A), using a column of oligo(dT)-cellulose (14), and examine the relative distribution of 32P-labeled primer amongst different size classes of these fragments and possibly deduce therefrom the site (or sites) at which primer is bound. As it happens, fragmentation of 35S RNA can occur prior to the harvesting of the virus and during purification (7). We have exploited this in the following experiment. The purified virus used was obtained from cells that had been uniformly labeled with [3H]uridine.

From the sedimentation analysis shown in Fig. 1, it can be seen that the [3H]-labeled poly(A)-containing RNA fragments range in sedimentation values up to about 35S. However, the majority of the 32P-labeled 4S RNA bound to these fragments is associated with large 28 to 35S species. Our interpretation of this finding is that the majority of primer binding sites are located at or near the 5’-terminus of the 35S RNA.

Also shown in Fig. 1 is the sedimentation analysis of the poly(A)-deficient RNA fragments. The distribution of bound primer among these fragments is consistent with the above-mentioned interpretation, but other interpretations are also possible.

Table 1 summarizes the relative content of 32P and [3H] in the various nucleic acid fractions obtained during the course of the experiment. The ratio 32P/[3H] should be directly proportional to the number of primer molecules per unit mass of RNA. Thus, relative to 70S RNA the poly(A)-deficient RNA has more primer and the poly(A)-containing RNA has less primer. However, poly(A)-containing RNA with a sedimentation value of 35S has the same ratio as the 70S. Again these data are in accord with the interpretation that the one primer binding site per 35S RNA is located at or near the 5’-terminus.

Also shown in Table 1 is the 32P/[3H] ratio for purified primer from this experiment. From this value, the size of 35S RNA (9,000 nucleotides...
[12]) and primer (75 nucleotides [6]), and the
\(^{32}P/^{3}H\) for 70S RNA, the number of primer
molecules bound per 35S RNA can be calculated.
The mean of four experiments was 1.04 ± 0.29. This value is in agreement with previous estimations (Taylor et al., Virology, in press; 9, 17).

To better estimate the location of the primer binding site with respect to the 5' terminus, we have analyzed the 35S RNA fragments on gels of 2.1% polyacrylamide (Fig. 2). This particular experiment was done with a different virus stock (strain B77, transformation defective). The markers of 18 and 28S rRNA were run on a separate gel. From their observed mobility and the formula mol wt = 1,550S\(^{2}\) of Spirin (18), the size scale as indicated was deduced. The

size distribution of the poly(A)-containing RNA (Fig. 2a) is such that 70% of the \(^{3}P\) is associated with species that are larger than 90% of the modal size. The absolute size values calculated could easily be in error in that they involve extrapolations of the Spirin formula. The size distribution of the poly(A)-deficient RNA is shown in Fig. 2b. The distribution of \(^{32}P\)-labeled primer amongst the \(^{3}H\)-labeled RNA is almost random, with a small but significantly higher frequency among smaller RNAs.

The studies described above show that amongst poly(A)-containing RNA the labeled primer is primarily associated with the largest RNA species. From this an interpretation has been adopted which says that the primer must be bound at or near the 5'-terminus. To further

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**Table 1. Analysis of radioactivity in aliquots of RNA fractions isolated during experiment described in Fig. 1**

<table>
<thead>
<tr>
<th>Aliquot of RNA fraction</th>
<th>(^{3}H) (counts/min)</th>
<th>(^{32}P) (counts/min)</th>
<th>(^{3}P/^{3}H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 70S RNA</td>
<td>4,580</td>
<td>265</td>
<td>0.068 ± 0.003a</td>
</tr>
<tr>
<td>Total poly(A)-deficient RNA</td>
<td>7,000</td>
<td>540</td>
<td>0.077 ± 0.003</td>
</tr>
<tr>
<td>Total poly(A)-containing RNA</td>
<td>4,100</td>
<td>103</td>
<td>0.025 ± 0.002</td>
</tr>
<tr>
<td>35S poly(A)-containing RNA*</td>
<td>2,940</td>
<td>163</td>
<td>0.055 ± 0.003</td>
</tr>
<tr>
<td>Purified primer*</td>
<td>37</td>
<td>243</td>
<td>6.6 ± 1.5</td>
</tr>
</tbody>
</table>

*This error represents the standard deviation arising from counting errors of both \(^{3}H\) and \(^{32}P\).

+ Fractions 6 and 7 in Fig. 1.

+ Primer was released from partially denatured 12 to 35S RNA by complete denaturation at 70 C as previously described (Taylor et al., in press; 5).

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![Fig. 2. Polyacrylamide gel electrophoresis of partially denatured viral RNA. Viral RNA was uniformly labeled with \(^{3}H\)thymidine (○), and bound primer molecules were specifically labeled with \(^{32}P\) (O). Electrophoresis was for 3.5 h at 4 mA/gel, with other details as described. Analyses of poly(A)-containing and poly(A)-deficient species are as shown in (a) and (b), respectively. The \(^{3}H\)-labeled 18 and 28S rRNA (arrows) were run in a separate gel. Using these markers and the formula of Spirin (18), the uppermost scale of RNA lengths was deduced. The virus used in this experiment was a transformation-defective variant of strain B77.](http://jvi.asm.org)
substantiate this interpretation we have also examined the effect of fragmentation induced after isolation of the poly(A)-containing RNA. Limited fragmentation was produced by heating at 45°C in a buffer at pH 10.0. At various times aliquots were removed and assayed for poly(A) content by a membrane filter (Millipore)-binding technique (15). The results (Fig. 3) indicate that with respect to the 3H-labeled RNA the 32P-labeled bound primer can more readily lose its poly(A)-containing property. This is again consistent with the primer being bound at or near the 5' terminus of the 35S RNA.

A more detailed analysis was made of poly(A)-containing RNA that had been fragmented for 9 min as described above. Figures 4a and b show the polyacrylamide gel analyses of this RNA before and after fragmentation. Clearly fragmentation has converted 32P-labeled primer and 3H-labeled RNA into faster migrating species. (Primer binding itself is not disrupted by this procedure since no primer is seen to migrate in the 4S region of the gel after fragmentation.) A larger sample of RNA after fragmentation was separated a second time on oligo(dT)-cellulose into poly(A)-containing and poly(A)-deficient fractions which were subsequently analyzed on polyacrylamide gels. In Fig. 4c, it can be seen that due to the fragmentation, the residual 3H-labeled poly(A)-containing RNA contains an increased amount of lower-molecular-weight species with respect to Fig. 4a. The 32P-labeled bound primer, however, is found only among the undegraded RNA species. It appears that the first nick, no matter where it occurs, removes the primer from the poly(A)-containing fragments. 3H-labeled RNA of the newly derived poly(A)-deficient species covers a range of up to almost full size, and the 32P-labeled bound primer is almost randomly distributed amongst these (Fig. 4d).

**Fig. 4.** Polyacrylamide gel electrophoresis of poly(A)-containing partially denatured viral RNA subjected to limited fragmentation. The materials used in this experiment are as in the legend to Fig. 2, with the poly(A)-containing RNA uniformly labeled with 3H (●) and the bound primer molecules specifically labeled with 32P (O). Fragmentation was induced as described in the legend to Fig. 3 by heating isolated poly(A)-containing RNA for 9 min at 45°C in 0.01 M Tris-0.01 M EDTA-0.025 M Na2CO3 (pH 10.0). The majority of the treated RNA was passed over oligo(dT)-cellulose to separate poly(A)-containing and poly(A)-deficient species. The electrophoretic analyses shown are of: (a) total poly(A)-containing RNA prior to fragmentation; (b) total after treatment; (c) poly(A)-containing component isolated after treatment; (d) poly(A)-deficient component isolated after treatment. Electrophoresis was for 1.75 h at 4 mA/gel. The 3H-labeled 18 and 28S rRNA (arrows) were run in a separate gel.

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*Fig. 3.* Induced fragmentation of poly(A)-containing partially denatured viral RNA. The materials used in this experiment are as in the legend to Fig. 2. Fragmentation was induced by heating at 45°C in 0.01 M Tris-0.01 M EDTA-0.025 M Na2CO3 (pH 10.0). At various times aliquots were removed and assayed for ability to bind to membrane filters (Millipore) (15). Data are expressed with respect to zero time, at which 100% of both the uniformly-labeled 3H RNA (●) and bound primer molecules specifically labeled with 32P (O) bound to the filter.
DISCUSSION

This paper relates our experience with the primer binding site on avian sarcoma virus genomes. We calculate that on the average there is one primer bound per 35S RNA in native 70S RNA, and also one primer per poly(A)-containing partially denatured 35S RNA. The majority of primer molecules are bound at or near the 5'-terminus of the RNA. We have obtained similar results with both a transforming and a nontransforming isolate of strain B77 avian sarcoma virus. The design of the present experiments is such that we cannot locate the primer site more precisely than by saying that at least 70% of the primers bound are on molecules that are larger than 90% of the modal size, assuming that this size represents intact RNA. There is a smaller but significant fraction (as high as 30%) of primer molecules that seems to be bound to smaller species (about 20 to 30S). It can be seen from Fig. 4c relative to Fig. 4a that the proportion of primer bound to poly(A)-containing RNA of this size neither increases nor decreases as a consequence of induced fragmentation of total poly(A)-containing RNA. One interpretation would be that there exists some poly(A)-containing RNA species, with primer bound at or near their 5'-terminus, that are only 20 to 30S in size.

It is interesting to speculate regarding the significance of the present results in terms of the process of in vivo viral replication. It must first be made quite clear that as yet there is evidence neither for nor against the proposal that the RNA primer, as studied in vitro, has a similar role in vivo. If this proposal were true, then because the primer is at or near the 5'-terminus, and since DNA synthesis proceeds toward the 5'-terminus of the template, it is necessary that during DNA synthesis the 5'-terminus of the RNA must come into juxtaposition with the 3'-terminus of the same 35S RNA or possibly with the 3'-terminus of another 35S RNA. The latter possibility is interesting for several reasons, one of which is that it suggests a role for the existence of the observed 70S RNA aggregate of 35S RNAs. Implicit in the former possibility is the formation of a circular intermediate in DNA transcription. Such an event might be the initial event in the formation of the double-stranded DNA circles that have been recently observed in vivo (10, 11) and which seem to be immediate precursors to integrated proviral DNA (11).

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LITERATURE CITED