Extent of Double Strandedness in Avian Myeloblastosis Virus RNA

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Received for publication 27 August 1974

The extent of double strandedness of avian myeloblastosis virus 70S RNA has been determined from fluorescence measurements of the intercalation of ethidium bromide. We have shown that 50% of the nucleotides of 70S RNA in solution are in a stable helical configuration. This value does not include small helical regions that are too unstable to permit intercalation of the dye. The avian myeloblastosis virus RNA as it exists within the virion has the same degree of helicity as the free 70S RNA. Heating the free 70S RNA to 55 or 70 C, followed by cooling, does not measurably change the degree of helicity; the subunits therefore have as much helicity as the parent molecule.

The RNA of oncornaviruses consists primarily of a high-molecular-weight species sedimenting in the 60 to 70S range (5, 6, 16). It is apparently composed of three or four large subunits which dissociate upon heating or on treatment with dimethylsulfoxide (1, 5, 15) or formamide (19). It is generally assumed that the subunits are held together by hydrogen bonds, giving rise to double-helical regions in the molecule. However, the extent of the helicity and the conformation of the 70S RNA are uncertain, both within the virion and after isolation.

Estimates of the helical content of the RNA have been made from its resistance to the specific single-strand nucleases (8, 9, 13, 14, 20). The main disadvantage of this procedure is that these enzymes have not been fully characterized. It is not known, for example, how long a helical region is required for resistance or what are the effects of internally melted or mismatched regions. To determine the helical content by a more precise procedure, we have used fluorescence spectroscopy. Ethidium bromide (EB) fluoresces intensely when it is intercalated between the base pairs of either RNA or DNA helices, but not when it binds to single-stranded regions (2, 11). We have been able to determine on this basis the helical content of isolated 70S RNA directly from fluorescence measurements. With the same methodology, we have also determined the helical content of the RNA as it exists within the virion. We have also shown that heating and then cooling of isolated 70S RNA does not lead to a decrease in helicity, implying that nearly all the helicity is within rather than between the subunits.

MATERIALS AND METHODS

Materials. Avian myeloblastosis virus (AMV) was supplied as plasma from infected chickens by Life Sciences, Inc. (St. Petersburg, Fla.).

Purified Reo virus double-stranded RNA (over 99% double-stranded) was kindly donated by M. Kinshnam (Roche Institute of Molecular Biology, Nutley, N.J.).

28S RNA isolated from rat liver was a generous gift of M. G. Hamilton. 4S RNA was purchased from General Biochemicals Div. (Mogul Corp., Chagrin Falls, Ohio). EB (grade B) was purchased from Calbiochem (Los Angeles, Calif.).

Virus and RNA isolation. Plasma was stored at -70 C and was thawed just prior to use. All operations were performed at 0 to 4 C. After thawing, the plasma was filtered through four layers of gauze and layered on top of a glycerol cushion, and virus was pelleted onto the cushion by centrifugation at 25,000 rpm for 1 h in an SW27 rotor. The virus was collected from the cushion, diluted threefold with buffer containing 0.01 M Tris-hydrochloride (pH 7.8), 0.15 M NaCl, and 0.01 M EDTA (TNE buffer), and layered over linear 20 to 50% (wt/vol) sucrose gradients prepared in TNE buffer. AMV was isopycnically banded at 105,000 × g in an SW27 rotor for 15 h at 4 C, at which time the virus band was collected, suspended in TNE buffer, and pelleted at 150,000 × g at 4 C for 30 min. 70S RNA was isolated by the method of Kacian et al. (12).

Methods. Fluorescence measurements were made with a Cary 50-026-900 differential recording spectrofluorimeter with front surface illumination at an angle of 23° between the exciting beam (546 nm) and the emitted light (602 nm). The illuminating light is broken up by a chopper and passed alternately through a rhodamine quantum detector (located in the sample compartment) and the sample. The signal displayed on the recorder is the ratio between these two signals, thus correcting for variation of light
intensity of the source. The ratio \( V \) of fluorescence intensity of bound EB to the intensity of free dye was determined for each RNA at 546 nm. For 28S rRNA and 4S RNA it was 50; for Reo and AMV RNA it was 70. No corrections for light scattering were found necessary at the concentrations and instrumental settings used in these experiments. Measurements were made on 1.1 ml of solution in a cuvette (2-mm path length). EB at a concentration of about \( 10^{-7} \) M was added to the cuvette by an automatic pipette capable of delivering 1 to 200 \( \mu \)l. The concentration of RNA in terms of phosphate was in the range of \( 1 \times 10^{-5} \) to \( 10 \times 10^{-4} \) M. The buffer was a mixture of 0.1 M NaCl and 0.05 M Tris (pH 7.8). Measurements were made at 25 C. The combined instrumental and experimental error was generally less than 2%.

RESULTS

The number \((n)\) of EB-binding sites per nucleotide phosphate, and the strength of binding \((k)\) are given by \( r/c = kn - kr \), where \( r \) is the number of EB molecules bound per nucleotide phosphate at a free EB concentration of \( c \). Clearly, \( n \) is the limiting value of \( r \) at infinite EB concentration. EB shows significant fluorescence only when it intercalates between base pairs. Theoretically, a maximum of one EB molecule can be bound per two phosphate groups (i.e., \( n = 0.5 \)), assuming that intercalation can occur between each pair of base pairs. Because of steric reasons, the actual number bound will always be substantially less than this.

The value of \( r \) is calculated from the intensity of fluorescence, \( I_r \), in the presence of RNA, as follows: \( r = I_r/(k \cdot P) \), where \( k = I_rV/c \), \( I_r \) is the intensity of fluorescence in the absence of RNA, the other conditions being identical to those under which \( I_r \) is measured. \( C_0 \) is the EB concentration (moles per liter), \( V \) is the ratio of intensities of bound and free EB, and \( P \) is the nucleotide phosphate concentration. (In a formal way, \( k \) is identical to an absorbance extinction coefficient.)

Reo virus and 28S rRNAs. Reo virus and 28S rRNAs have been chosen as models of known structure to provide a basis for estimating the degree of helicity of 70S RNA. For this reason, we have determined complete EB-binding curves for both RNAs (Fig. 1). The value of \( n \) (the number of EB molecules bound per phosphate at saturation) for Reo virus RNA is 0.044, indicating that one EB molecule is intercalated in the 100% double-stranded Reo virus RNA per 11.4 base pairs, or one EB per 23 phosphate groups. The value of \( n \) for 28S rRNA is 0.012, indicating that 28S rRNA intercalates 26% as much EB as the completely helical Reo RNA. The data for both nucleic acids are represented by a linear plot showing unique values for \( k \) (a measure of the strength of binding) and \( n \) in each case. Table 1 summarizes the \( k \) values and the number of phosphate groups per EB molecule (number is equal to 1/\( n \)) for all RNAs examined.

AMV RNA. The results for the binding of EB to isolated 70S AMV RNA are shown in Fig. 1 (curve 1). The data are fit best with a straight line, which indicates that there is only one set of binding sites with a single binding constant. This means that all intercalation sites are essentially indistinguishable. The value for \( n \) is 0.022, or 1 EB per 45 phosphate groups. The same binding curve was obtained whether the RNA was used directly from the sucrose gradient or first precipitated with alcohol and redissolved.

To test the possibility that structural changes might occur or that factors might be lost during the isolation of the 70S RNA, the same experiment was carried out with the intact virus and with Nonidet P-40-disrupted virions. The NP-40 dissociates most of the protein from the RNA, leaving a "core" structure containing some protein. The results should show whether the mere act of disruption gives rise to impor-
Isolated tant inent the contribution determine 5, 7, the non-70S RNA Thus, we agreement with the results in groups the total RNA irrelevant RNA this in of their contribution com-prise the extent of value for contribution to low rRNA 28S and again are in NP-40-disrupted virions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$n$</th>
<th>No. of phosphate/*</th>
<th>$k \times 10^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated 70S RNA</td>
<td>0.022</td>
<td>45</td>
<td>2.7</td>
</tr>
<tr>
<td>Disrupted virion</td>
<td>0.025</td>
<td>40</td>
<td>1.7</td>
</tr>
<tr>
<td>70S RNA</td>
<td>0.022</td>
<td>45</td>
<td>ND*</td>
</tr>
<tr>
<td>70S RNA in virion c</td>
<td>0.022</td>
<td>45</td>
<td>2.1</td>
</tr>
<tr>
<td>Isolated 70S RNA (pre-heated to 55°C)</td>
<td>0.022</td>
<td>45</td>
<td>2.1</td>
</tr>
<tr>
<td>Isolated 70S RNA (pre-heated to 70°C)</td>
<td>0.022</td>
<td>45</td>
<td>2.1</td>
</tr>
<tr>
<td>28S rRNA</td>
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<td>84</td>
<td>1.9</td>
</tr>
<tr>
<td>Reo virus RNA</td>
<td>0.044</td>
<td>22</td>
<td>5.5</td>
</tr>
</tbody>
</table>

a The number of phosphate groups per EB molecule is the reciprocal of $n$, the value of $r$ at the abscissa in Fig. 1 and 2.

b $k$ is obtained by dividing the ordinate intercept by $n$.
c 10 μl of a suspension of AMV (1.6 mg of protein per ml, 0.65 M Tris-0.1 M NaCl [pH 7.8]) was added to 1.1 ml of $2.47 \times 10^{-7}$ M EB in the same buffer. The final 70S RNA phosphate concentration was $1.2 \times 10^{-4}$ M.
d ND, Not determined.

tant structural changes affecting the intercalation of EB. However, before doing this we had to determine the contribution to the intensity of fluorescence of the other RNAs which are present in the virion in addition to the 70S RNA: the 4, 5, 7, 18, and 28S RNA components. Most of the non-70S RNA is of the 4S type; we found that one EB intercalates per 75 phosphate groups in 4S RNA (data not shown). This is in agreement with the results of Tao et al. (18). Thus, we were able to correct for the small increment in the intensity of fluorescence due to this irrelevant RNA (which amounts to 20% of the total RNA in the virus [7]). The extent of intercalation in 28S rRNA is also low, as discussed above. We did not determine the binding of EB to the 5, 7, or 18S RNAs, but since these RNAs comprise only about 8% of the total RNA, their contribution to the total fluorescence is low and can be neglected. We estimate this contribution to be about 2%, assuming that the extent of binding is about the same as for the 4S and 28S RNAs.

The results for the binding of EB to 70S RNA in NP-40-disrupted virions (Fig. 1, curve 2) again are fit best by a straight line, indicating that there is only one set of binding sites. The value for $n$ (0.025), however, is slightly higher than that for isolated 70S RNA. We do not know the significance of this difference, if any.

In the determination of a complete binding curve, increasing amounts of EB are added to a fixed concentration of RNA and then are extrapolated in both directions to infinite EB concentration and zero EB concentration to obtain $n$, the number of binding sites, and $k$, the binding constant. In the case of the undisrupted virus, the RNA content of the virion is low, so that the amount of RNA required for binding studies at low EB concentrations produces a turbidity too high to permit meaningful measurements. That is, nearly all of the fluorescence is lost by light scattering. It is thus impossible to determine $k$. However, the value of $n$ can be determined with a small quantity of virus and a large excess of EB. This procedure is tantamount to a direct determination of the number of EB molecules bound at infinite EB concentration. The value of $n$ determined for 70S RNA within the virion is 0.022, identical to that for isolated 70S RNA (Table 1). This indicates that the secondary structure of the 70S RNA does not change upon isolation.

**Heated 70S RNA.** Heating of 70S RNA results in two specific changes (3). At 55°C this ionic strength the molecule dissociates into 35S subunits, which are still active as primer-templates in assays in vitro. At 70°C, virtually all of the 70S dissociates into 35S subunits and apparently small (<10S) primer RNAs are dissociated from these subunits with a concomitant loss of template activity. 70S RNA samples were heated to each of these temperatures in the cuvette and were cooled quickly in ice to minimize reannealing. The binding of EB to the heat-treated RNAs is shown in Fig. 2, where it is evident that the curves for the samples heated either to 55°C or 70°C are identical to that for unheated 70S RNA. Any destruction of helicity at 55 or 70°C is therefore largely reversible.

**DISCUSSION**

We have determined the binding constants and the number of sites at which intercalation of EB occurs for AMV 70S RNA in various physical states, using fluorescence measurements to indicate intercalation. It has been established that fluorescence results from intercalation of the EB molecule between base pairs present in a helical structure, and not from binding to single-stranded nucleic acids (11). Our results are summarized in Table 1. The calculation of these parameters does not depend on any assumptions. The fact that all the data are expressible by straight lines shows that there is only one type of binding site, associated
with the fluorescence phenomenon. This has been reported earlier both for DNA and RNA (11).

By comparing the number of binding sites for 70S RNA to that for Reo virus RNA, which is completely double-stranded, we calculate the degree of helicity of the AMV 70S RNA to be 50%. Because the molecular weights of these RNA molecules are high, differences in molecular weight do not affect the binding properties (11). However, this value may be a minimum if there are also double-stranded regions in the 70S molecule which are too short to bind EB. We have assessed this situation by comparing the degree of helicity of 28S rRNA calculated by different methods. The fluorescence measurements reported here indicate that 26% of 28S RNA is double stranded by comparison with Reo virus RNA. However, it has been shown by various other physical methods that about 60% of this RNA is helical (17), with regions that have been estimated to be 4 to 17 base pairs long (4). We have shown (L. F. Cavaliere, unpublished data) that helical regions equal to or larger than 10 base pairs bind EB. We deduce then that helical regions which are about 4 to 7 base pairs long do not bind EB or do so very weakly, since 58% of the helicity of 28S rRNA is not observed by fluorescence. Any similar short helical regions in 70S RNA would not be registered in the fluorescence measurements. The 50% helicity observed must therefore correspond to longer stretches which are not transient, thus implying a degree of complementarity which is significant with regard to the information content of the RNA. Nearly all the helicity must be internal rather than between subunits, since it is reversible, as shown by the heating results. We are investigating this structural aspect of the subunits, since it is undoubtedly important in the role of the subunits as RNA messengers, as well as in their reverse transcription.

ACKNOWLEDGMENTS

I thank Elizabeth Carroll for expert technical assistance, Barbara Hatch Rosenberg for helpful discussions, and Stuart Marcus and Mukund Modak for isolation of the AMV RNA.

This investigation was supported by Public Health Service grants 08748-09A and 08748-09B from the National Cancer Institute and Atomic Energy Commission contract AT (11-1)-5521. AMV was prepared under Public Health Service contract NOICP33291 of the Virus Cancer Program of the National Cancer Institute.

LITERATURE CITED


