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Primer Recognition by Avian Myeloblastosis Virus RNA-Directed DNA Polymerase

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Tryptophanyl-tRNA was specifically labeled at the 3′ end with [^{3}H]tryptophan and cleaved in half with RNase under denaturing conditions, and the 3′ half was shown to hybridize exclusively at the 5′ end of avian myeloblastosis virus RNA. The RNA-dependent DNA polymerase of avian myeloblastosis virus is capable of efficiently binding the 3′ half of the primer molecule.

The RNA genome of avian tumor viruses serves as a template for RNA-directed DNA polymerase, which initiates DNA synthesis using a 4*S* RNA primer (4, 6, 7). In the case of avian myeloblastosis virus (AMV), this primer has been shown to be identical to cellular tryptophanyl-tRNA (8). The primer is found hybridized to the RNA genome in the virus. The hybrid structure is located within 200 nucleotides of the 5′ end of each of the 35*S* RNA subunits (1). The 3′ end of the tRNA is involved in this hybrid structure from the penultimate residue extending 16 nucleotides into the molecule (3, 5). Thus, the stem region and a portion of the pseudouridine loop structure are involved in binding to the template RNA. RNA-directed DNA polymerase has the ability to specifically bind tryptophanyl-tRNA, selecting the primer molecule from among a mixture of RNA species (10). It has recently been proposed that RNA-directed DNA polymerase, like aminoacyl-tRNA synthetases, recognizes tRNA by the structure of the stem region and that the enzyme may serve as an agent in the positioning and formation of the template-primer hybrid (3). To test the specificity of RNA-directed DNA polymerase for the tRNA structure, we have enzymatically cleaved $tRNA^{trp}$ into approximately half-molecules. We have found that RNA-directed DNA polymerase is capable of efficiently binding the 3′ half of $tRNA^{trp}$. The intact stem region is not required for recognition by the enzyme. The sequences of portions of the anticodon loop, the pseudouridine loop, and the 3′ terminus of the tRNA remain as likely structural clues for primer recognition.

To follow the binding of $tRNA^{trp}$ to RNA-directed DNA polymerase, we have acylated the molecule with [^{3}H]tryptophan. Wherever possible, in the manipulations described, conditions have been chosen to minimize hydrolysis of the labile aminoacyl linkage (13). The aminoacyl-tRNA synthetase mixture and tRNA were purified from fresh chicken liver as described by Yang and Novelli (14). The tRNA was charged with [^{3}H]tryptophan, using the conditions of Harada et al. (8), and stopped by adjusting to 0.3 M sodium acetate (pH 5.2), extracting twice with phenol, and precipitating with ethanol. Portions of the [^{3}H]tryptophanyl-$tRNA^{trp}$ were cleaved into roughly half-molecules by digestion with pancreatic RNase. Optimal digestion conditions were 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, 80% formamide, with an enzyme-substrate ratio of 1:1,000. The reaction was carried out at room temperature for 1 min, stopped by dilution into 10 volumes of 0.3 M sodium acetate (pH 5.2), and extracted three times with phenol. The solution was precipitated with 2 volumes of ethanol, and the precipitate was dissolved in 0.3 M sodium acetate (pH 5.2), incubated with 1 mg of iodoacetic acid per ml for 15 min at room temperature, and again precipitated with ethanol. When run in 98% formamide-10% acrylamide gels, the cleaved tRNA migrates as a single peak, with an estimated size of 45 nucleotides relative to 4*S* and 5*S* RNA (Fig. 1). About 50% of the input label is recovered in this peak. The remainder is lost due to cleavage of the 3′ terminus of $tRNA^{trp}$ to ethanol-soluble fragments.

The binding of $tRNA^{trp}$ and cleaved $tRNA^{trp}$ to RNA-directed DNA polymerase was measured by the assay of Panet et al. (10). AMV and RNA-directed DNA polymerase were obtained from J. Beard through the Viral Biology Branch of the National Cancer Institute. About 10% of either the intact or cleaved $tRNA^{trp}$ elutes in the void volume of the G-100 column due to binding to RNA-directed DNA polymerase (Fig. 2). The cleaved $tRNA^{trp}$ is clearly smaller than intact tRNA, although they can-

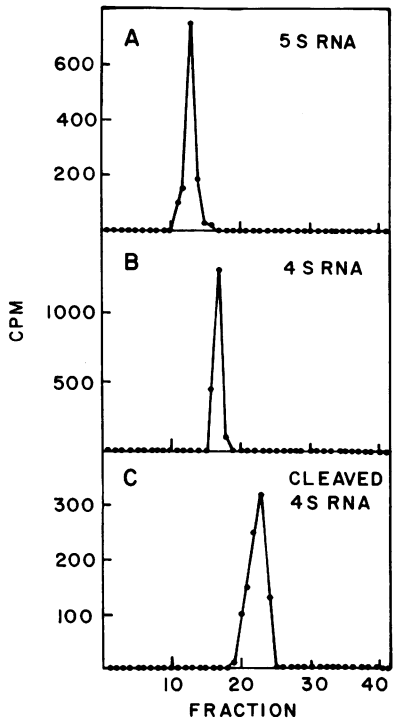

FIG. 1. *Polyacrylamide gel electrophoresis of [^{3}H]tryptophanyl-tRNA before and after cleavage with RNase. Ten percent acrylamide gels were made up in formamide essentially according to the method of Staynov et al. (11), except that the supporting electrolyte solution was 0.02 M sodium acetate, 0.01 M sodium citrate, pH 4.3. The RNA samples were layered under a column of 97.5% formamide, 0.02 M sodium acetate, 0.01 M sodium citrate (pH 5.5) and subjected to electrophoresis at 4 mA/gel for 4 h. The 5S RNA marker was purified from [^{3}H]uridine-labeled HeLa ribosomes by electrophoresis.*

not be completely resolved by column chromatography. However, the cleaved tRNA preparation contains no intact labeled molecules and, thus, the binding to RNA-directed DNA polymerase must be due to binding of cleaved tRNA (Fig. 1). At the pH of the binding reaction (8.3), 24 to 35% of the tryptophan is lost by deacylation of the aminoacyl-tRNA and elutes from the column after the tRNA fraction.

As has recently been described by Cordell et al. (3), the 3′ half of cleaved tRNA^{trp} retains the ability to hybridize with AMV RNA. Furthermore, we have found that cleaved tRNA^{trp} retains the property of preferential hybridization to the 5′ terminus of AMV RNA. AMV RNA (70S) was prepared by dissociating AMV with 1% sodium dodecyl sulfate and 0.1% dextran-SO_4 and isolated on 15 to 30% sucrose gradients. The 70S RNA was denatured in 50% formamide at 60°C for 2 min, and the denatured RNA was centrifuged on 15 to 30% sucrose gradients at 28,500 rpm for 16 h at 20°C in an SW40 rotor. The average size of the RNA fragments obtained in this manner was 10S, with less than 5% intact 35S RNA molecules. The RNA smaller than intact molecules was pooled and precipitated in ethanol. The AMV RNA fragments were redissolved and fractionated by oligo(dT)-cellulose column chromatography [oligo(dT)-cellulose kindly supplied by T. G. Wood], as described by Merkel et al. (9). Approximately 12% of the AMV RNA bound to oligo(dT)-cellulose, which is close to the predicted amount based on average 10S molecules if each intact 35S AMV molecule had poly(A) at its 3′ end (i.e., most, if not all, 3′ ends were isolated). The poly(A)-containing material, representing 3′ terminal fragments, was pooled, that lacking poly(A), representing 5′ terminal fragments, was also pooled, and the two pools were ethanol precipitated. The hybridization of

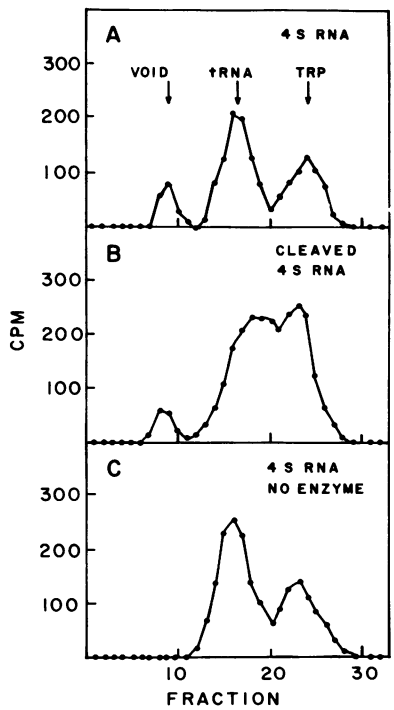

FIG. 2. *Binding of [^{3}H]tryptophanyl-tRNA to RNA-directed DNA polymerase. Intact and RNase-cleaved ^{3}H-labeled tRNA^{trp} were bound to saturating concentrations (150 U/ml) of RNA-directed DNA polymerase under the conditions of Panet et al. (10) and chromatographed on 25-cm Sephadex G-100 columns.*

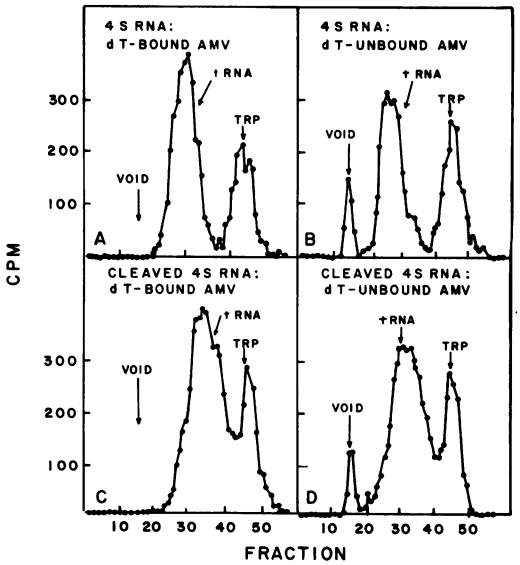

FIG. 3. *Hybridization of intact and RNase-cleaved [^{3}H]tryptophanyl-tRNA to the 3′ and 5′ ends of AMV RNA. The experimental details of the hybridization to 3′ and 5′ ends of AMV RNA are described in the text. The hybridization mixture was chromatographed on G-100 Sephadex in 2 × SSC, pH 6.0.*

[^{3}H]tryptophanyl-tRNA^{trp} to AMV RNA fragments was carried out in 2× SSC (pH 4.3) (SSC = 0.15 M NaCl + 0.015 M sodium citrate), 50% formamide for 16 h at 33°C (13). The final concentration of total chicken tRNA was 3 mg/ml in the reaction. Under these conditions of hybridization, the tRNA is in excess of the AMV binding sites and tRNA drives the reaction. Hybridization was terminated at 10 times the estimated $R_0t_{1/2}$. (Hybridization carried out to 20 times the estimated $R_0t_{1/2}$ did not increase the amount of tRNA hybridized.) Both intact and cleaved tRNA hybridized with the 5′ terminal fragments, as determined by exclusion of the hybrid on Sephadex G-100 columns (Fig. 3). No hybrid was detected with the poly(A)-containing 3′ terminal fragments of AMV RNA. These results confirm the observation of others (1, 2, 12) that the major binding site for tRNA^{trp} is present at the 5′ terminus of AMV RNA. The 3′ half of cleaved tRNA^{trp} is, however, as effective in forming hybrids at the 5′ end of the AMV RNA as the intact molecule.

It has been suggested that RNA-directed DNA polymerase positions the primer on AMV RNA and facilitates the formation of a stable hybrid (3). Our initial attempts to demonstrate this reaction in vitro have been unsuccessful. AMV RNA 5′ terminal fragments have been added to the RNA-directed DNA polymerase-tRNA^{trp} complex formed as described in the legend to Fig. 2. The complex formed is sensitive to 1% sodium dodecyl sulfate, as would be expected if the protein is necessary for stability, but not if an AMV RNA-tRNA^{trp} hybrid was formed.

Our results indicate that the secondary structure of the stem region of tRNA^{trp} is not necessary for the binding of the tRNA by RNA-directed DNA polymerase or the formation of hybrids with AMV RNA. RNA-directed DNA polymerase is able to efficiently recognize tRNA^{trp} lacking a double-stranded stem region. Our experiments agree with recently published studies of the location and orientation of the primer molecule on the AMV template. The mechanism of the formation of a full-length DNA transcript, using as a primer tRNA^{trp} located close to the 5′ end of its template, remains a major question.

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