Proc. Nat. Acad. Sci. USA
Vol. 73, No. 4, pp. 1329–1332, April 1976
Microbiology

Evidence for circularization of the avian oncornavirus RNA genome during proviral DNA synthesis from studies of reverse transcription *in vitro*

(RNA tumor virus/RNA-directed DNA synthesis/nucleic acid hybridization/nucleic acid circularization)

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Communicated by Maurice B. Visscher, February 5, 1976

ABSTRACT **The RNA-directed DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase EC 2.7.7.7) of avian oncornaviruses requires a tryptophan tRNA ($tRNA^{Trp}$) primer molecule located close to the 5′ end of the viral RNA genome for the initiation of DNA synthesis *in vitro*. In this communication we demonstrate that the DNA product, transcribed from avian myeloblastosis virus (AMV) 35S RNA containing only $tRNA^{Trp}$ as primer, is located also at the 5′ end of the RNA genome. More importantly, we demonstrate that these 5′ terminal DNA transcripts contain nucleotide sequences complementary to the 3′ end of the genome. We have interpreted these results to mean that the 3′ and 5′ termini of the AMV 35S RNA genome become juxtaposed with each other either before or immediately after DNA synthesis has begun. These results are discussed in regard to the mechanism for synthesis of the circular forms of oncornavirus proviral DNA.**

Initiation of DNA transcription of the avian oncornavirus 70S RNA genome occurs on a 4S RNA molecule exhibiting structural and aminoacylation properties of tryptophan tRNA ($tRNA^{Trp}$) (1–6). The recent localization of this primer RNA species at or near the 5′ end of the avian oncornavirus genome (ref. 7; K. A. Staskus, M. S. Collett, and A. J. Faras, submitted for publication) raises several interesting questions regarding both the limitations and restrictions of reverse transcription *in vitro* (8) and the precise mechanism of proviral DNA synthesis *in vivo*. Regarding the latter issue, if the $tRNA^{Trp}$ primer RNA required for reverse transcription *in vitro* is also responsible for the initiation of proviral DNA synthesis *in vivo*, then, in order for the complete transcription of the avian oncornavirus genome to occur, either the 5′ and 3′ termini of the 35S RNA subunit species comprising the 70S RNA complex are maintained in proper end-to-end alignment with each other, or the 5′ terminus of a single 35S RNA subunit species comes in juxtaposition with its own 3′ terminus (7). Although the oncornavirus genome is ultimately converted *in vivo* into a circular form of provirus DNA representing a complete transcript of 35S RNA (9, 10), the mechanism by which this circularization event occurs is presently unknown. In this communication we present data from our studies on the mechanisms of RNA-directed DNA synthesis *in vitro* suggesting that the 5′ and 3′ termini of individual 35S RNA subunits become juxtaposed either before or immediately after DNA synthesis has begun.

MATERIALS AND METHODS

Reagents and Virus. The sources and preparation of most of the pertinent materials have been previously described (1, 3, 11). Avian myeloblastosis virus (AMV) (in plasma) and purified AMV RNA-directed DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase EC 2.7.7.7) were obtained from Dr. J. W. Beard of Life Sciences, Inc., St. Petersburg, Fla., through the auspices of the Viral Cancer Program of the National Cancer Institute. The virus was concentrated by pelleting and subsequently purified according to published procedures (12). In some instances the AMV DNA polymerase was further chromatographed on CM-Sephadex and/or Sephadex G-100 to reduce the levels of RNase activity present in some of the preparations.

Purification of Viral RNA and Various Size-Classes of Poly(A)-Containing Fragments of the Viral Genome. RNA was extracted from virus with sodium dodecyl sulfate/phenol at room temperature and subsequently fractionated into 70S RNA and free, low-molecular-weight RNA by rate-zonal sedimentation (12). 35S RNA subunits, free of any 70S-associated (70S-a) low-molecular-weight RNA including primer RNA, were prepared by rate-zonal sedimentation subsequent to treatment of viral 70S RNA with 95% (vol/vol) dimethylsulfoxide (12). Although we commonly refer to these RNA subunits as 35S RNA, the subunit species obtained from these preparations of virus generally represent a heterogenous size-class of RNA ranging from 4 to 40 S. For this reason no further fragmentation of the AMV RNA was necessary to obtain a broad range of size-classes of poly(A)-containing fragments. The poly(A)-containing fragments were purified by affinity chromatography on poly(U)-Sepharose as previously described (K. A. Staskus, M. S. Collett, and A. J. Faras, submitted for publication).

Reconstitution of Template Activity. Reconstitution of template activity of viral 35S RNA was performed as follows unless otherwise stated in the legends to the figures. AMV 35S RNA or poly(A)-containing fragments (200 μg/ml) were annealed with primer RNA purified as previously described (12) for 30 min at 75° in 0.6 M NaCl, 0.02 M Tris·HCl (pH 7.5), and 0.01 M EDTA in 5 μl volumes in sealed glass capillary pipettes. Subsequent to incubation the samples were cooled to room temperature and diluted into 50 μl reaction mixtures to a final RNA concentration of 4 μg/ml. Enzymatic reactions routinely contained 0.5 unit of enzyme per ml, 0.1 M Tris·HCl (pH 8.1), 0.01 M $MgCl_2$, 2% (vol/vol) 2-mercaptoethanol, 50 μM unlabeled deoxynucleoside triphosphates (dTTP, dATP, dCTP), 5 μM ^{3}H-labeled dGTP (15–25 Ci/mmol). Unless otherwise indicated, the reactions were incubated at 37° for 2 hr and analyzed for perchloric-acid-precipitable radioactivity as described previously (13).

RESULTS

The 5′ location of the primer site on the avian oncornavirus RNA genome was determined by structural studies involv-

Abbreviation: AMV, avian myeloblastosis virus.

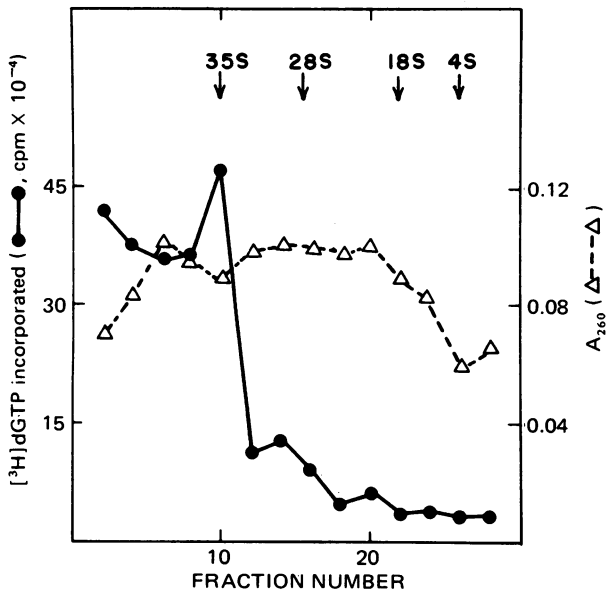

FIG. 1. Localization of the primer site by reconstitution of template activity of the AMV RNA genome. Poly(A)-containing fragments, ranging from 200 nucleotides to genome length in size and prepared as described in the *Materials and Methods*, were subjected to rate-zonal sedimentation in 15–30% sucrose in a Spinco SW 50.1 rotor at 50,000 rpm for 225 min at 4° in order to separate the various size-classes from one another. Individual fractions were assayed for A_{260} (open triangles). Exactly 1 μg of RNA from each fraction was reannealed with 0.15 μg of purified primer RNA under conditions described in the *Materials and Methods*. Template activity of reconstituted template-primer complexes (closed circles) was determined by incubation with the purified AMV DNA polymerase as described previously (12). Each fraction has been corrected for the endogenous activity observed after the fragmented RNA was reannealed in the absence of primer RNA. Arrows represent the position of ^{3}H-labeled Rous sarcoma virus 35S RNA (from virus harvested at 4 hr intervals from infected cell cultures) and chick 28S, 18S, and 4S RNA run in a parallel sucrose gradient as sedimentation references.

ing radiolabeled primer RNA (ref. 7; K. A. Staskus, M. S. Collett, and A. J. Faras, submitted for publication). We have confirmed these studies by functionally localizing the primer site on the AMV RNA genome by reannealing purified unlabeled primer RNA to fragmented AMV RNA and determining the template activity of the reconstituted template-primer complexes with the purified AMV DNA polymerase. The approach is basically similar to our previous localization studies (K. A. Staskus, M. S. Collett, and A. J. Faras, submitted for publication) and makes use of the poly(A) sequences at the 3′ terminus of AMV RNA as a means of localizing the primer site with respect to the 3′ end of the genome. The procedure consists of first separating the various size-classes of poly(A)-containing fragments by rate-zonal sedimentation and then analyzing the ability of the fragments to function as an active template-primer complex subsequent to reannealing with primer RNA (12). Typical results of such an experiment are presented in Fig. 1. Of the various size-classes of poly(A)-containing fragments ranging between 200 nucleotides and genome length, only the largest RNA molecules (≥35 S, i.e., 10,000 nucleotides) are capable of reannealing to primer RNA and becoming an active template-primer complex for the AMV DNA polymerase. These results indicate that the primer RNA is located close to or at the 5′ end of the genome and that a functional template-primer complex is formed only if poly(A)-containing AMV fragments approaching genome length in size are reannealed with primer RNA. Since no radiolabeled primer is capable of reannealing to fragments less than 9000 nucleotides in length (K. A. Staskus, M. S. Collett, and A. J. Faras, submitted for publication), we can rule out the possibility that primer anneals to all size classes of fragments but functions only with genome length RNA.

The 5′ end location of the primer site on the AMV genome implies that the bulk of the DNA transcripts synthesized in response to $tRNA^{Trp}$ as primer on the AMV genome should also be found at the 5′ end of the genome. To directly prove this contention we have reannealed purified primer RNA to poly(A)-containing fragments of the AMV genome and incubated the resultant template-primer complexes in a brief enzymatic reaction containing the AMV DNA polymerase to synthesize DNA. The DNA products of the reaction were then analyzed to determine the size-class of RNA fragments with which they were associated. As can be seen in Fig. 2A the bulk of the DNA transcripts sediment only with poly(A)-containing fragments of near genome-length in size, indicating their association with the 5′ end of the AMV genome. Denaturation of the DNA product-poly(A)-containing RNA complex prior to sedimentation releases DNA that remains at the top of the sucrose gradient, indicating that these DNA transcripts are small and that the sedimentation profile presented in Fig. 2A is not due to the presence of long DNA transcripts (14). Furthermore, all of these DNA transcripts band at a density intermediate to that of RNA and DNA in Cs_2SO_4, indicating their covalent association with primer RNA (data not shown). The exact size of these DNA transcripts released from template and primer RNA by alkaline hydrolysis ranged between 50 and 150 nucleotides as demonstrated by their relative electrophoretic mobilities in polyacrylamide gels (Fig. 3).

In an effort to determine whether these DNA transcripts synthesized on the 5′ end of the AMV RNA genome are complementary to the 3′ end of the genome, the DNA in the 35S region of the sucrose gradient in Fig. 2A was treated with alkali to remove RNA and then annealed to all of the various size-classes of poly(A)-containing fragments of the AMV genome. As can be seen in Fig. 2B the DNA transcripts recovered from the 5′ terminus of the AMV genome hybridized equally as well to the large- and small-size classes of poly(A)-containing fragments, indicating that these 5′ terminally located transcripts contain sequences complementary to the 3′ terminus of the AMV genome.

Since the DNA transcripts synthesized under these reaction conditions contain several discrete size-classes which can easily be resolved from one another by electrophoresis in 10% polyacrylamide gels (Fig. 3), an experiment was performed to determine the minimum size-class of DNA transcripts containing the nucleotide sequences complementary to the 3′ termini. The DNA from the three major regions of the gel was eluted and hybridized to total poly(A)-containing fragments and poly(A)-containing fragments less than 4500 nucleotides in length (<28 S). From the data presented in Table 1 it appears that even the smallest size-class of DNA transcripts (ca. 50 nucleotides) contains nucleotide sequences complementary to the 3′ terminus of the AMV RNA genome.

Extending these latter observations even further, we have attempted to determine whether the deoxynucleotide sequence immediately adjacent to the primer molecule is also complementary to the 3′ end of the AMV genome. In these particular experiments the initial DNA sequence, which is

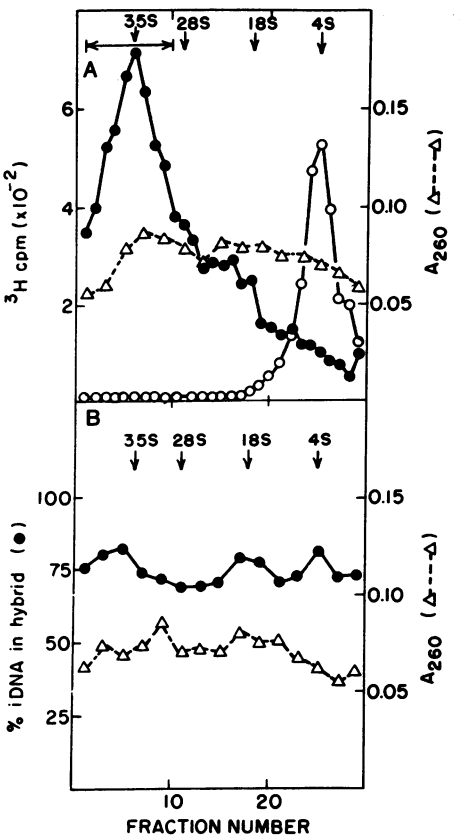

FIG. 2. Localization of tRNA^{Trp} primer RNA-initiated DNA at the 5′ terminus of the AMV genome and analysis of nucleotide sequences complementary to this DNA. *Panel A.* Purified, unlabeled primer RNA was reannealed to a sample of poly(A)-containing RNA fragments, diluted 6-fold with TE buffer (0.01 M Tris·HCl, pH 7.4, 0.01 M EDTA), and heated at 60° for 3 min to disrupt aggregates of poly(A)-containing fragments that form during the reannealing (A. Faras, unpublished observation). This mixture was then incubated in an enzymatic reaction containing all four deoxynucleoside triphosphates as follows: unlabeled dTTP and dATP at 60 μM, [^{3}H]dCTP at 10 μM, and [^{3}H]dGTP at 3 μM, purified AMV DNA polymerase, and 32 μg/ml of bentonite, to prevent template degradation by traces of RNase present in the DNA polymerase preparation (18). Enzymatic synthesis was terminated after 15 min at 37°, at which time the reaction mixture was treated with sodium dodecyl sulfate/Pronase, phenol extracted, and ethanol precipitated. The precipitate was collected by centrifugation and resuspended in 0.01 M Tris·HCl, pH 7.4, 0.01 M EDTA. The sample was then divided into two aliquots. One aliquot was heated to 100° for 45 sec (○) and the other was left untreated (●). The samples were analyzed by rate-zonal sedimentation in 15–30% sucrose in a SW 50.1 rotor at 50,000 rpm for 225 min at 4°. The sedimentation profiles of both samples are superimposed in the figure for comparative purposes. The arrows represent the positions of ^{32}P-labeled Rous sarcoma virus 35S RNA and chick cell 28S, 18S, and 4S RNA included in the gradients. The A_{260} profile of the poly(A)-containing fragments used in the reannealing reaction is also displayed (Δ).

Panel B. Fractions 1–10 in the sucrose gradient described in panel A were pooled, treated with alkali to remove all RNA (0.6 M NaOH, 37° for 3 hr), neutralized, and ethanol precipitated. These ^{3}H-labeled initial DNA (iDNA) transcripts were then hybridized to various size-classes of viral poly(A)-containing RNA fragments separated from one another by rate-zonal sedimentation as described in panel A. The A_{260} profile of the poly(A)-containing RNA fragments is indicated by the open triangles. Hybridization was performed under conditions of RNA excess as follows. ^{3}H-labeled DNA (1500 cpm, 0.1 ng), 5 μg of yeast RNA, and 0.3 μg of the poly(A)-containing RNA fragments from each fraction in 25 μl of 0.6 M NaCl, 0.01 M Tris·HCl, pH 7.4, 0.01 M EDTA were sealed in 40 μl glass capillary pipettes and incubated for 50 hr at 68°. The extent of hybridization was measured by digestion with S_1 nuclease (19). Intrinsic S_1 resistance of the ^{3}H-labeled DNA was 22%. The specificity and fidelity of hybridization of these DNA transcripts to AMV RNA was also determined. No increase in S_1 resistance was observed when the homopolymer poly(adenylic acid) was substituted for the AMV RNA fragments in the hybridization mixtures, indicating that the DNA transcripts were hybridizing to heteropolymeric regions of the RNA. Furthermore, the hybridization was specific, since the DNA transcripts would only hybridize to avian oncornavirus RNA. The fidelity of hybridization between the DNA transcripts and AMV RNA was also determined by denaturation studies of the hybrid structures formed. The melting temperature, T_m, (88°, 0.3 M NaCl) of the hybrids was identical to that of total cDNA·AMV 70S RNA hybrid complexes, indicating little, if any, mismatching during the hybridization reactions.

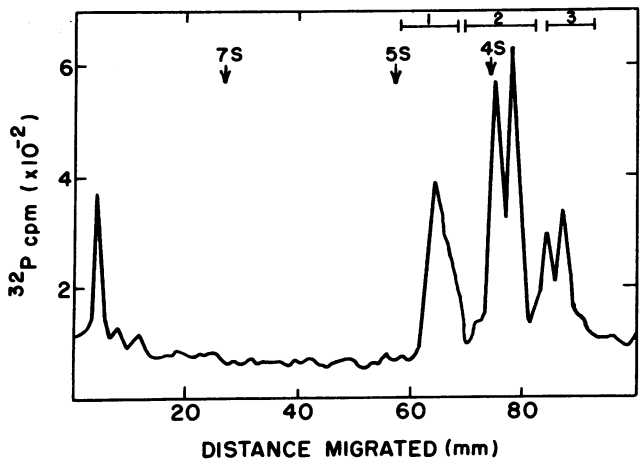

FIG. 3. Polyacrylamide gel electrophoresis of initial DNA transcripts. ^{32}P-Labeled DNA transcripts, synthesized and isolated from sucrose gradients as described in Fig. 2, were alkali treated and then subjected to electrophoresis in 10% polyacrylamide gels (0.6 × 10 cm) at 5 mA per gel for 4.5 hr at room temperature. Cerenkov radiation was determined in individual gel slices (1 mm). Arrows indicate the positions of RNA markers run in a parallel gel.

the heptanucleotide (A-A-T-G-A-A-G)$_{OH}$ (15, 16), can be specifically labeled with [^{3}H]deoxynucleoside triphosphates if deoxycytosine triphosphate is omitted from the reaction mixtures during enzymatic synthesis. Furthermore, since this heptanucleotide is incapable of forming a stable RNA·DNA hybrid with AMV RNA because of its short length (M. S. Collett, K. A. Staskus, and A. J. Faras, unpublished observations), the additional maneuver of elongating this labeled oligonucleotide was performed by subsequent addition of a large excess (1000-fold) of all four unlabeled deoxynucleotide triphosphates to the enzymatic reaction mixtures. The resultant DNA transcripts containing the radiolabeled heptanucleotide were capable of forming a stable hybrid structure with the AMV genome. From the data in Table 1 it is apparent that the labeled heptanucleotide portion of these DNA transcripts is capable of forming an S_1 nuclease-resistant hybrid structure with the 3′ terminus of the AMV genome, suggesting that the sequences complementary to the deoxynucleotides immediately adjacent to the primer molecule located at the 5′ end of the AMV genome are complementary to the 3′ end of the genome.

DISCUSSION

The data presented in this communication indicate that the DNA transcripts initiated upon the tRNA^{Trp} primer RNA present at the 5′ end of the AMV oncornavirus RNA genome

Table 1. Hybridization of various size-classes of DNA transcripts to fragmented poly(A)-containing AMV RNA

DNA transcripts	RNA	% Duplex
Total DNA*	None	11.8
	Total	85.2
	<28 S	96.0
Region 1 DNA*	None	0.7
	Total	94.0
	<28 S	92.0
Region 2 DNA*	None	23.6
	Total	78.0
	<28 S	84.5
Region 3 DNA*	None	0
	Total	92.0
	<28 S	95.0
Heptanucleotide-labeled DNA†	None	21.5
	Total	65.7
	<28 S	76.0

* DNA, synthesized and subsequently alkali treated as described in the legend to Fig. 2, was subjected to electrophoresis in a 10% polyacrylamide gel (Fig. 3). The DNA was eluted from the regions of the gel indicated by the bars in Fig. 3 as previously described (14), and then hybridized to either total poly(A)-containing fragments of the AMV genome or fragments less than 4500 nucleotides in size (<28 S) and tested for S_1 nuclease resistance as described in Fig. 2B. As a control for the amount of DNA reassociation occurring during hybridization, a sample of DNA was incubated under the hybridization conditions in the absence of AMV RNA.

† Heptanucleotide-labeled DNA was synthesized in reconstituted reactions as follows. Reaction mixtures, containing 6–8 μg/ml of reconstituted AMV RNA·primer complexes (primer was annealed to poly(A)-containing AMV RNA as described in the *Materials and Methods*), three ^{3}H-labeled deoxynucleoside triphosphates, dTTP, dATP, and dGTP at 3.3 μM, and purified AMV DNA polymerase, were incubated for 15 min at 37°. After the incubation period a large excess of all four unlabeled deoxynucleoside triphosphates (2 mM) was added to the reactions, which were incubated at 37° for 60 additional minutes. The reactions were terminated by the addition of sodium dodecyl sulfate/Pronase, phenol extracted, ethanol precipitated, and subsequently alkali treated. Hybridization was performed as described in footnote*.

contain nucleotide sequences complementary to the 3′ terminus of the genome. One possible interpretation of these observations is that the 5′ and 3′ termini of the avian oncornavirus genome are juxtaposed and transcription therefore proceeds from the primer RNA to the 5′ end and continues on the 3′ end of the genome. Since only 35S RNA or fragments thereof were employed in these studies it is clear that these observations were not the result of transcription of the 5′ end of one 35S subunit species and the 3′ end of another in the 70S RNA complex. One major difficulty with the aforementioned interpretation of the data, however, is that the AMV DNA polymerase would have to bridge a gap between the 5′ and 3′ termini of the genome RNA in order for transcription to occur on the 3′ end. Since no DNA polymerase studied to date is capable of continuing DNA synthesis after it reaches a nick or gap in the template, it would have to be postulated that an RNA ligase (17) is required to join the ends of the genome prior to transcription of the 3′ terminus.

Another interpretation of the data presented in this communication is that the DNA transcripts are synthesized on the 5′ end of a linear molecule but that the genome RNA is terminally redundant and therefore the 3′ end contains nucleotide sequences complementary to the DNA transcribed from the 5′ end. In this model it is possible that the RNA genome circularizes shortly after DNA synthesis has begun, and might involve the initial DNA product at the 5′ end of the genome associating with the terminally redundant complementary nucleotide sequences at the 3′ end (M. S. Collett, J. M. Coffin, and A. J. Faras, submitted for publication). We favor this model of circularization, since no requirement for the AMV DNA polymerase to bridge gaps or RNA ligase to join ends of the genome would be necessary for the synthesis of an intact, circular DNA copy. If this model is correct then the presence of the primer RNA close to the 5′ end of the genome RNA would serve an important and required function in the formation of a circular RNA·DNA hybrid intermediate in the synthesis of oncornavirus proviral DNA.

We thank S. Kanellos and P. Wilkie for excellent technical assistance and K. Staskus for her help in some of the analyses. We also thank Drs. P. G. W. Plagemann, R. L. Erikson, and J. F. Zissler for their editorial assistance and L. Honza for typing this manuscript. We are indebted to Dr. J. Beard, Dr. M. Chirigos, and the Office of Program Resources and Logistics, Viral Cancer Program, Division of Cancer Cause and Prevention, National Cancer Institute for supplying us with valuable reagents, without which many of these studies could not have been performed. This investigation was supported by Public Health Service Research Grant no. CA 18303-01 from the National Cancer Institute. M.S.C. is currently a predoctoral fellow also supported by this grant.

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