Transcription of *in Vitro* Polyadenylated Ribonucleic Acid with Reverse Transcriptase†

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ABSTRACT: A method has been developed for adding a short poly(A) stretch to the 3′ end of non-poly(A) containing RNA for the purpose of providing a binding site for an oligo(dT) primer, thus allowing initiation of transcription by AMV reverse transcriptase. Although *in vitro* polyadenylated RNA was found to promote the synthesis of DNA with greatly increased efficiency, the nature of the cDNA was found to be dependent upon the structure of the RNA template. cDNA transcribed from readenylated mouse globin mRNA from which the original poly(A) sequence had been removed was found to contain somewhat increased amounts of nonhybridizable material, but was otherwise identical with cDNA transcribed from native globin mRNA. Polyadenylated preparations of 28S rRNA, on the other hand, were found to promote the synthesis of large quantities of cDNA which failed to hybridize to the template RNA and contained a high content of poly(thymidylic acid). The results suggest that under the reaction conditions employed, a primer region alone may not be sufficient to allow completely faithful copying of natural RNA by reverse transcriptase, but that either primary or secondary structural features of the template exert considerable effect.

The use of reverse transcriptase from avian myeloblastosis virus (AMV) to synthesize DNA complementary to a variety of RNA templates has become a useful and widely used tool in the study of a number of metabolic processes related to the expression of genetic information in eukaryotes. The fact that the enzyme requires a "primer" for initiation of DNA synthesis, however, has limited the number of different types of RNA templates which may be copied using reverse transcriptase. Specifically, the template must contain a natural double-stranded primer region, or else contain a region to which a complementary sequence can be hybridized—for example, a terminal poly(A) stretch (Baltimore and Smoler, 1971; Duesberg *et al.*, 1971). Although most natural eukaryotic mRNAs have been shown to contain a terminal poly(A) tract, the mRNAs coding for histone proteins, for instance, do not (Adesnik and Darnell, 1972) and are thus presumably inactive as a template for reverse transcriptase.

Since a number of enzymatic activities, from both prokaryotic and eukaryotic sources, have been reported to catalyze the addition of poly(A) to the 3′ terminus of RNA molecules (Edmonds and Abrams, 1960; August *et al.*, 1962; Hardy and Kurland, 1966; Terzi *et al.*, 1970; Twu and Bretthauer, 1971; Ohasa *et al.*, 1972; Ohasa and Tsugita, 1972; Tsiapalis *et al.*, 1973; Winters and Edmonds, 1973), it should be possible to utilize such an enzyme to polyadenylate RNA *in vitro* and thus produce a suitable template for transcription by AMV reverse transcriptase. We have isolated a poly(A) polymerizing activity from high salt washes of *Escherichia coli* ribosomes (Hardy and Kurland, 1966) and have used it to polyadenylate both mouse globin mRNA from which the original poly(A) tract had been removed by polynucleotide phosphorylase digestion, and preparations of 28S ribosomal RNA from mouse embryos. Mouse globin mRNA was selected as a test system for two reasons: (1) the poly(A) tract can be selectively removed, leaving the rest of the molecule virtually intact (Williamson *et al.*, 1974), and (2) the cDNA synthesized from native mouse globin mRNA by reverse transcriptase is well characterized (Verma *et al.*, 1972; Kacian *et al.*, 1972) and provides a useful control. We report here on the properties of DNA synthesized from such templates by AMV reverse transcriptase.

Materials and Methods

Preparation of Poly(A) Polymerase. Poly(A) polymerase was isolated from mid-log *E. coli* cells strain MRE 600 (MRE, Porton) by a modification of the procedure of Hardy and Kurland (1966). All operations were performed at 0–4° unless otherwise indicated; 30 g of cells was partially thawed in 25 ml of TSM buffer (0.01 M Tris–0.003 M succinic acid–0.01 M $MgSO_4$ (pH 8.0)) containing 10 μg/ml of DNase I (Worthington); 1.7 ml of 10 × TSM and 60 g of glass beads were added, the cells were disrupted in an MSE top drive homogenizer for 12 min at high speed, and the mixture was centrifuged for 30 min at 25,000*g*. The supernatant was adjusted to 30 μg/ml in puromycin (Sigma), incubated for 15 min at 32°, and diluted to 100 ml with ice-cold TSM; 21 g of solid $(NH_4)_2SO_4$ was then added to give 27% saturation, and the mixture was stirred for 3 min. To the supernatant obtained from centrifuging at 20,000*g* for 10 min, an additional 21 g of $(NH_4)_2SO_4$ was added to give 55% saturation and the precipitated crude ribosomes were pelleted by centrifuging at 20,000*g* for 10 min. The pellet was dissolved in 100 ml of TSM and the $(NH_4)_2SO_4$ fractionation repeated two more times as before except that the fraction precipitating between 27 and 50% saturation was taken in the second cycle and that precipitating between 27 and 46% saturation was taken in the third cycle. The final pellet was dissolved in 25 ml of TSM and dialyzed against 1 l. of TSM for 2 hr, 1 l. of fresh buffer for 4 hr, and finally against another liter of fresh buffer overnight. The dialyzed sample was then centrifuged for 2 hr at 150,000*g* in an MSE 8 × 25 Al fixed-angle rotor. The ribosomal pellet was dissolved in 40 ml of TSM and centrifuged for an additional 90 min at 150,000*g*. The poly(A) polymerase activity was dissociated

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from the ribosomes obtained from the second high speed spin by dissolving the pellet in 40 ml of TSM containing 0.6 M $(NH_4)_2SO_4$ and pelleting the ribosomes at 150,000*g* for 3 hr. The top 30 ml of supernatant was removed and adjusted to 40% $(NH_4)_2SO_4$ saturation by the addition of 6.8 g of $(NH_4)_2SO_4$ followed by stirring for 10 min. The precipitate was collected by centrifuging at 20,000*g* for 10 min and dissolved in 30 ml of TSM–0.6 M $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ fractionation was repeated exactly as before and the final precipitate dissolved in 1.5 ml of TSM–0.6 M $(NH_4)_2SO_4$ containing 2 mM dithiothreitol.

Insoluble material was removed by a low-speed spin and the supernatant applied to a 1.6 × 46 cm Sephadex G-200 column equilibrated in TSM–0.6 M $(NH_4)_2SO_4$–2 mM dithiothreitol. The enzyme was eluted with the same buffer at a flow rate of 10 ml/hr; 1.0–1.5-ml fractions were collected and those between the void volume and 1 bed volume assayed for poly(A) polymerase activity by the standard assay. Fractions showing peak activity were pooled and the enzyme was precipitated by addition of solid $(NH_4)_2SO_4$ to 40% saturation. The precipitated enzyme was collected by centrifugation at 20,000*g* for 10 min and dissolved in 0.5 ml of TSM–0.6 M $(NH_4)_2SO_4$–2 mM dithiothreitol. An equal volume of 100% glycerol was added and the preparation stored at −20°. The enzyme is stable in this medium and maximum activity is retained for a period of several months.

Standard Assay for Poly(A) Polymerase Activity. The standard assay mix contained in a final volume of 200 μl: 10 mM Tris, 3 mM succinic acid (pH 8.0), 20 mM $MgSO_4$, 16 mM $MnCl_2$, 0.5 mM ^{3}[H]ATP (Radiochemical Centre, Amersham) (10 Ci/mol), 100 μg of *E. coli* rRNA, and either 10 μl of concentrated enzyme or 50 μl of each G-200 column fraction. Incubation was for 20 min at 37°. The reaction was stopped by the addition of 1.0 ml of 0.1 M KH_2PO_4, 100 μg of bovine serum albumin, plus 2.0 ml of ice-cold 7.5% Cl_3CCOOH containing 0.1% sodium pyrophosphate. The precipitate was collected by centrifugation, washed three times with 3-ml volumes of 7.5% Cl_3CCOOH–0.1% pyrophosphate, and dissolved in 1.0 N NaOH for counting; 1 unit of enzyme is defined as that amount of protein catalyzing the incorporation of 0.1 nmol of AMP into acid-insoluble material in 20 min at 37°. Counts from complete reaction mixtures containing no enzyme were subtracted to determine net radioactivity incorporated.

Polyadenylation of RNA for Reverse Transcriptase Reaction. Conditions for polyadenylation of RNA were as described in the standard assay conditions with the following exceptions: 10 μg of RNA plus 25 units of poly(A) polymerase were used in a 200-μl reaction mix and the incubation was performed for 10 min at 37° rather than 20 min. Unlabeled ATP was used. At the end of the incubation period, 1 ml of NETS buffer (0.1 M NaCl–0.01 M Tris–0.001 M EDTA–0.5% sodium lauryl sulfate (pH 7.4)) plus 100 μg of *E. coli* rRNA as carrier were added and the mixture was extracted once with an equal volume of phenol–chloroform (1:1 v/v) saturated in NETS buffer. The aqueous phase was adjusted to 3% in NaCl and the RNA precipitated at −20° with 4 volumes of absolute ethanol. The precipitated RNA was collected by centrifugation, dissolved in 0.5 ml of NETS buffer, and chromatographed on a 0.9 × 30 cm Sephadex G-50 column equilibrated in NETS buffer. RNA eluting in the excluded volume was recovered by ethanol precipitation and dissolved in 100 μl of glass-distilled H_2O for use. Siliconized glassware, rinsed with dilute diethylpyrocarbonate, was used for all procedures to eliminate exogenous nuclease activity.

Reverse transcriptase was prepared from avian myeloblastosis virus (kindly supplied by Dr. J. W. Beard) as described by Kacian *et al.* (1971) and stored in 50% glycerol–50% 0.3 M potassium phosphate (pH 8.0) at −20°. The activity of the enzyme solution was 100 units/ml (Kacian *et al.*, 1972).

Preparation of [^{3}H]cDNA. [^{3}H]Thymidine-labeled cDNA was synthesized by incubating at 37° for 2 hr a mixture (0.25 ml) containing 13.4 μM [^{3}H]dTTP (15 Ci/mmol; Radiochemical Centre, Amersham), 200 μM dCTP, 200 μM dATP, 200 μM dGTP, 40 μg/ml of actinomycin D, 20 μg/ml of oligo(dT_{17}) (PL Biochemicals), 1.4–8 μg/ml of template RNA, 200 μg/ml of bovine serum albumin, 50 mM KCl, 5 mM magnesium acetate, 10 mM dithiothreitol, 50 mM Tris-HCl (pH 8.2), and 50 μl of reverse transcriptase (Harrison *et al.*, 1972a,b). cDNA was isolated, then fractionated on linear 4–11% (w/w) alkaline sucrose gradients as described previously (Harrison *et al.*, 1972b). Fractions containing cDNA of desired molecular weights were pooled, neutralized, and precipitated with carrier *E. coli* DNA (20 μg/ml) with 2 volumes of ethanol at −20°. The DNA precipitate was washed with ethanol, dried, and dissolved in sterile distilled water.

Hybridization of cDNA and RNA. Appropriate amounts of cDNA and RNA and 10 μg of *E. coli* RNA in sterile water were mixed and lyophilized. The mixture of nucleic acids was redissolved in 20 μl of 0.5 M NaCl, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.5 ml of EDTA, and 50% formamide (Fluka) (pH 6.8), sealed in siliconized capillaries, and incubated at 43°. All salt solutions were passed through Chelex-100 resin (Bio-Rad Laboratories) then treated with diethylpyrocarbonate and autoclaved before mixing with formamide.

Nuclease Assay for Hybridization. After incubation, each capillary was flushed out with nuclease buffer consisting of 0.07 M sodium acetate (pH 4.5), 0.14 M NaCl, 2.8 mM $ZnSO_4$, and 14 μg/ml of heat-denatured mouse DNA; 0.25 ml of this mixture was incubated with 100 μl of S_1 nuclease (prepared from takadiastase as described by Sutton (1971)) at 37° for 2 hr. The activity of the enzyme solution was sufficient to ensure that all single-stranded DNA was degraded completely within 1 hr under these conditions. A portion of the incubation mixture was taken to determine the total radioactivity. Another portion was acidified (0.5 N $HClO_4$) at 4° after addition of carrier mouse DNA (5 μg) and bovine serum albumin (50 μg), then centrifuged at 2500 rpm for 15 min. A portion of the supernatant fluid was taken to measure the acid-soluble radioactivity.

Isolation of RNA. 28S rRNA was prepared by standard phenol extraction techniques from purified 60S ribosomal subunits of 14-day mouse embryos. The preparations were estimated to be 95% 28S rRNA by polyacrylamide gel electrophoresis. Mouse globin mRNA was prepared from reticulocyte polyribosomes from mice made anaemic by phenylhydrazine injection. Isolation of purified mRNA was performed by chromatography of total polysomal RNA on oligo(dT)-cellulose columns (Aviv and Leder, 1972).

Deadenylation of Mouse Globin mRNA. 3'-Terminal poly(A) tracts were removed from mouse globin mRNA by polynucleotide phosphorylase digestion (Williamson *et al.*, 1974). Deadenylated RNA was isolated from the total digest by chromatography on poly(T)-cellulose columns.

Analysis of Poly(T) Content of cDNA. The poly(T) content of [^{3}H]TTP-labeled cDNA was estimated by polyacrylamide gel electrophoresis of diphenylamine–formic acid digests of the cDNA (Burton, 1967). Following electrophoresis on 5%

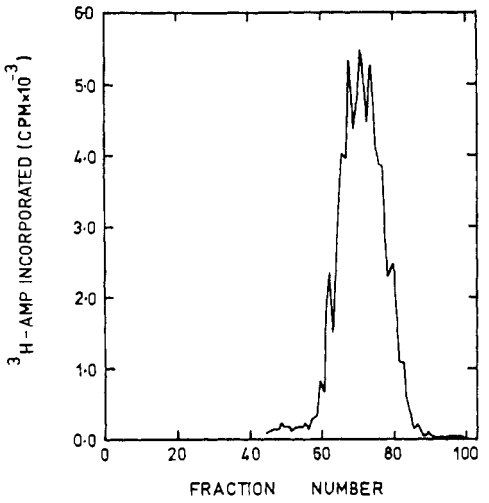

FIGURE 1: Elution profile of *E. coli* poly(A) polymerase from Sephadex G-200. Conditions of chromatography were as described in Materials and Methods. The void volume corresponds to fraction number 45 and the bed volume to fraction 100.

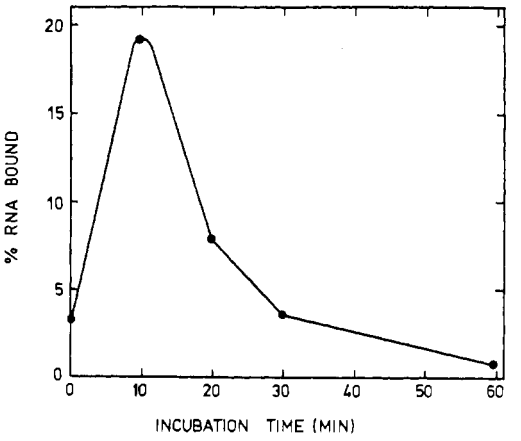

FIGURE 2: Binding of polyadenylated RNA to poly(U)-Sepharose, time course of reaction. [^{3}H]Uridine-labeled 18S rRNA (3.3 × 10^4 cpm/μg) (24 μg) was incubated with 42 μg of poly(A) polymerase in a total reaction volume of 600 μl; 100-μl aliquots were withdrawn at indicated times, diluted to 0.5 ml with NETS buffer, and applied to 1-ml poly(U)-Sepharose columns. Unbound RNA was eluted with NETS while bound RNA was eluted with 90% formamide elution buffer.

gels, the gels were fractionated and the radioactivity per slice was determined.

Miscellaneous. Oligo(dT)-cellulose was purchased from Collaborative Research, Waltham, Mass.

Protein concentrations were determined by the method of Lowry *et al.* (1951).

Sedimentation coefficients of cDNA were estimated from alkaline sucrose gradients data by the method of McEwen (1967). Molecular weights were calculated from the corresponding $s_{20,w}$ values using the relationship $s_{20,w} = 0.0528M^{0.400}$ (Studier, 1965).

Results

Characteristics of E. coli Poly(A) Polymerase. Figure 1 shows an elution profile of poly(A) polymerase from Sephadex G-200. The activity elutes as a slightly heterogeneous peak about midway between the void volume and bed volume of the column.

Table I lists the activity of three different preparations of poly(A) polymerase under standard reaction conditions, both in the presence and absence of added RNA. In all cases, the primer-independent activity was less than 4% of that observed in the presence of RNA indicating that the enzyme preparations have a virtually complete requirement for exogeneous primer.

Previous studies utilizing zone centrifugation and nearest neighbor analysis have demonstrated that the reaction product of *E. coli* poly(A) polymerase is a short chain of poly(A) (approximately 20 nucleotides long) attached to the 3′ terminus of the primer RNA (Hardy and Kurland, 1966). Figure 2 shows the results of an experiment in which [^{3}H]-uridine-labeled 18S rRNA was reacted with poly(A) polymerase using unlabeled ATP as a substrate. The per cent of the labeled RNA which was polyadenylated at different incubation times was determined by chromatographing samples of the reaction mixture on poly(U)-Sepharose columns. As illustrated, there was an initial sharp increase to a level of almost 20% of the primer RNA bound after 10 min, followed by a progressive decrease until after 60 min of incubation, the level of binding had fallen below the zero time level. The most likely explanation for these results is that small quantities of contaminating ribonuclease activity are introducing breaks in the polyadenylated RNA, resulting in a progressive decrease in the [^{3}H]uridine counts bound to the column during extended reaction conditions. For this reason, all polyadenylation reactions were limited to 10-min incubation.

Template Activity of Polyadenylated RNA. Table II lists the template activities of polyadenylated RNA and nonpolyadenylated controls when assayed in a standard reverse transcriptase reaction mix. In both cases, polyadenylated RNA exhibited large increases in template activity as compared to nonpolyadenylated RNA. The deadenylated mouse globin mRNA exhibited less than 1% of the template activity of the native mouse globin mRNA as would be expected if virtually all of the poly(A) sequences had been removed. The *in vitro* readenylated globin mRNA gave an increase to

TABLE I: Primer Requirement of *E. coli* Poly(A) Polymerase.

Preparation No.	pmoles of AMP Incorporated (+) RNA	(−) RNA
1.	616.7	1.6
2.	2268.8	61.4
3.	980.0	35.4

TABLE II: Reverse Transcriptase Template Activity of Polyadenylated RNA.

Template	pmoles of [^{3}H]TMP Incorporated 250 μl^{-1} μg^{-1} of RNA
Globin 9S mRNA	
1. Native	888.9
2. Deadenylated	5.76
3. Readenylated	112.9
28S rRNA	
1. Native	4.08
2. Polyadenylated	117.7

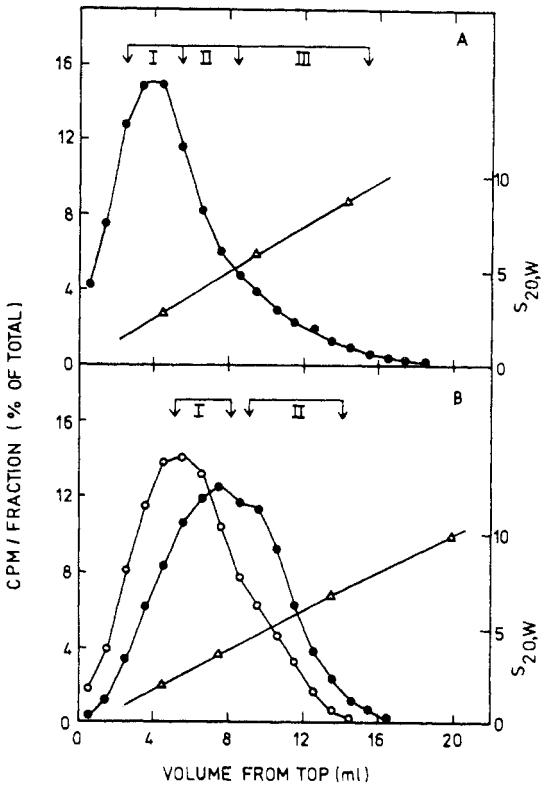

FIGURE 3: Alkaline sucrose gradient centrifugation of [^{3}H]TMP-labeled cDNA. Run conditions were as described in Materials and Methods: panel A, 28S rRNA–cDNA; panel B; native and readenylated globin mRNA–cDNA ((●) native, (○) readenylated); (△) $s_{20,w}$. Fractions were pooled for further study as indicated by the brackets.

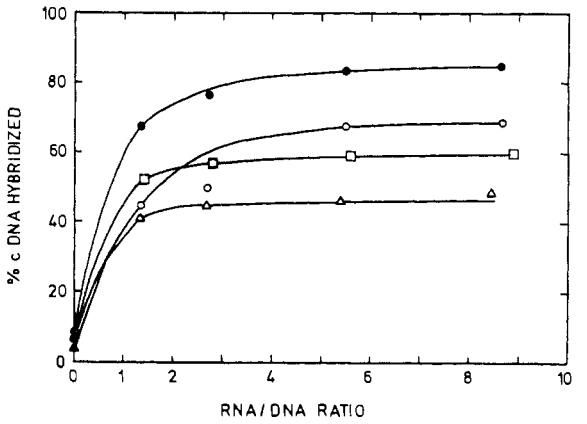

FIGURE 4: Hybridization of native and readenylated globin mRNA–cDNA. Hybridization mixtures were assembled as described in Materials and Methods. Each capillary contained 10 μg of *E. coli* RNA, 10,000 cpm of the indicated cDNA fraction and the appropriate amount of the respective template RNA. Incubations were performed for a time period sufficient to allow reactions involving the lowest RNA/DNA ratio to go to completion: (●) native globin cDNA fraction II; (○) native globin cDNA fraction I; (□) readenylated globin cDNA fraction II; (△) readenylated globin cDNA fraction I.

approximately 13% of the template activity of the native RNA. Moreover, both readenylated globin mRNA and adenylated preparations of 28S rRNA are equally efficient templates for reverse transcriptase.

Size of cDNA Transcripts. The molecular weight of cDNA was determined by sedimentation in alkaline sucrose gradients. As shown in Figure 3, cDNA synthesized from *in vitro* polyadenylated RNAs using [^{3}H]TTP as label exhibited a main peak sedimenting at 2.5–3.0 S, corresponding to a single-strand molecular weight of approximately 2×10^4. Under identical conditions cDNA transcribed from native globin mRNA exhibited a peak center molecular weight of 4×10^4. In all cases, significant amounts of much higher molecular weight material were present. The decrease in the average molecular weight of cDNA transcribed from readenylated globin mRNA as compared to that synthesized from the native template is most likely a result of the low level nuclease activity contaminating the poly(A) polymerase preparations.

Hybridization of cDNA. In order to test the ability of the cDNAs to hybridize to the RNA from which they were transcribed, fractions of different molecular weights were obtained by pooling selected regions from alkaline sucrose gradients, as illustrated in Figure 3, and titrated with increasing quantities of native RNA. Hybridization curves for high and low molecular weight cDNA transcribed from native and readenylated globin mRNA templates are shown in Figure 4. The main difference was a reduction in the saturation values for the transcripts from the readenylated RNAs relative to the transcripts from native RNA. In both instances, the low molecular weight cDNA hybridizes to a lower saturation value than does the higher molecular weight cDNA. The nature of the nonhybridizable component of native globin cDNA is unclear but has been observed and reported previously (Harrison *et al.*, 1974).

The saturation values obtained with cDNAs transcribed from the polyadenylated 28S rRNA preparation are listed in Table III. It is clear that a very different situation exists. Not only are the saturation values much lower, but little difference is observed in the hybridization of low and high molecular weight fractions of cDNA.

Poly(T) Content of cDNAs. A possible explanation for the decreased percentage of hybridizable cDNA in transcripts synthesized from *in vitro* polyadenylated templates as compared to those made from *in vivo* polyadenylated RNA might be that there is an increased percentage of ^{3}H-labeled poly(T) in such transcripts, possibly arising from a small amount of free poly(A) in the reverse transcriptase reaction mixture and insufficient amounts of oligo(T) to suppress its transcription completely. If this were the case, one would expect an enrichment of this material in the lower molecular weight fraction of cDNA, and correspondingly decreased amounts in the cDNA of higher molecular weight. Figures 5a and b show the

TABLE III: Hybridization of cDNA Fractions.

cDNA Fraction	Av Mol Wt[a]	% cDNA Hybridized at Saturation[b]
28S I	1.6×10^4	17.1
28S II	6.5×10^4	23.0
28S III	2.3×10^5	20.5
Readen. 9S I	2.5×10^4	43.9
Readen. 9S II	1.2×10^5	51.7
Native 9S I	2.5×10^4	58.9
Native 9S II	1.2×10^5	79.8

[a] Average mol wt refers to the mol wt of the cDNA in the center of the brackets indicated in Figure 3. [b] All values are corrected by subtracting the % hybridization obtained to *E. coli* rRNA (2–5%).

gel patterns obtained from a diphenylamine–formic acid digest of cDNA fractions I and II transcribed from native globin mRNA. The large, more rapidly migrating peak of radioactivity corresponds mainly to free nucleotides while the smaller, slower migrating shoulder represents [^{3}H]TMP-labeled polypyrimidine tracts. The fact that a percentage of the digested cDNA approximately equal to that found in the slower migrating peak also binds to poly(A)-Sepharose columns suggest that this material represents mainly poly(T). Figures 5c and d show the patterns obtained from cDNA fractions I and II synthesized from the *in vitro* readenylated globin mRNA while Figures 5e and f show the patterns obtained from low and high molecular weight cDNA transcribed from the *in vitro* polyadenylated 28S rRNA preparation. The much larger proportion of poly(T) in these cDNAs compared to those in the globin messenger cDNAs is immediately apparent.

Discussion

While considerable differences have been shown to exist in the poly(T) content of the various cDNAs investigated, it is quite clear that the quantitative differences alone are insufficient to account for the observed differences in hybridization properties. With respect to the two globin cDNAs, the actual percentages of poly(T) in the transcripts are virtually identical and probably play only a small part in the decreased level of hybridization of cDNA made from the readenylated template relative to that synthesized from native globin mRNA. Since approximately 20% of the native globin cDNA does not hybridize under the conditions employed, it is possible that there are simply increased amounts of this material in the cDNA transcribed from readenylated globin mRNA templates.

In the case of cDNA transcribed from polyadenylated preparations of 28S rRNA, our data do not exclude the possibility that it is a minor component RNA which is polyadenylated and not the 28S RNA. However, we consider this possibility unlikely since at most 5% of the subunit RNA is not 28S RNA, while by comparison with readenylated globin mRNA, at least 13% has been polyadenylated sufficiently to act as a template for reverse transcriptase. It is quite likely, however, that at least some of the polyadenylated molecules represent fragments of the original 28S rRNA resulting from the low level nuclease activity in the poly(A) polymerase preparations.

Regardless of the origin of the template, it is quite clear that the cDNA transcripts exhibit properties quite different from those transcribed from either of the globin mRNA preparations. Not only does this cDNA exhibit greatly increased quantities of poly(T), there is only a marginal reduction in poly(T) content in cDNA averaging 430 nucleotides in length as compared to cDNA averaging only 36 nucleotides in length. The bulk of this poly(T), then, must be covalently linked to the cDNA and not free. In addition, the poly(T) stretches in both the low and high molecular weight cDNA are of approximately the same size as judged by their almost identical electrophoretic mobilities in polyacrylamide gels. These data suggest that the high molecular weight cDNA contains short poly(T) stretches dispersed in some manner throughout the length of the molecule. Such a structure could well account for the observation that the high molecular weight cDNA does not hybridize significantly better than does the low molecular weight fraction—a situation which most certainly does not exist with either of the globin cDNAs. In

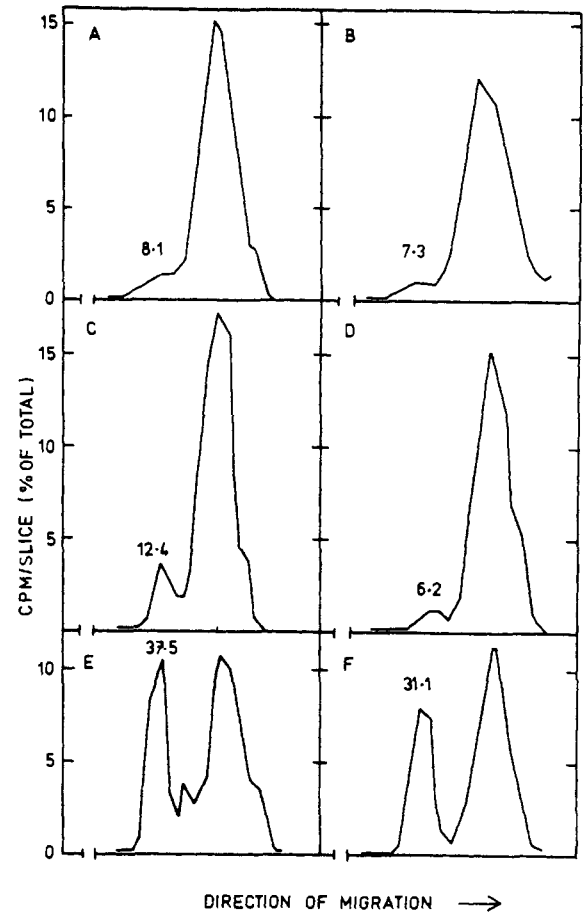

FIGURE 5: Poly(T) content of cDNA. Diphenylamine–formic acid digestion and polyacrylamide gel electrophoresis were performed as described in Materials and Methods. Each digest contained 30,000 cpm of [^{3}H]TMP-labeled cDNA and 50 μg of unlabeled calf thymus DNA as carrier. The percentage of the total digest radioactivity running as poly(T) is indicated over the first peak in the diagrams: (A) native globin cDNA fraction I; (B) native globin cDNA fraction II; (C) readenylated globin cDNA fraction I; (D) readenylated globin cDNA fraction II; (E) 28S cDNA fraction I; (F) 28S cDNA fraction III.

considering how such a structure could arise, it seems clear that it must be a function of the template RNA itself rather than an artifact introduced by the poly(A) polymerase reaction. This is demonstrated by the fact that cDNA synthesized from *in vitro* readenylated globin mRNA exhibited a poly(T) content and distribution virtually identical with that of cDNA made from a native globin mRNA template. The actual mechanism giving rise to such a structure is more difficult to envisage, but could involve a slippage phenomenon whereby the reverse transcriptase initiates, transcribes in a short distance, and then slips back and reinitiates, this time faithfully copying the poly(A) region as well since the original poly(T) primer is now covalently attached to the end of the growing cDNA chain. The end result would be a covalently linked series of repeat units, each of which begins with a short poly(T) stretch. Although it is not clear what might produce such a situation, a high degree of secondary structure in the template RNA is an obvious candidate.

The important conclusion to be drawn from the data presented here is that a high percentage of the cDNA transcribed from a readenylated globin mRNA template does not differ significantly from that transcribed from native globin mRNA. This approach may therefore prove useful for other RNAs which do not occur naturally polyadenylated. It is necessary,

however, to recognize the complication arising from nuclease activity in the preparations of poly(A) polymerase as well as the influence of template structure upon the fidelity of cDNA synthesized by reverse transcriptase. Since the preparation and use of the poly(A) addition enzyme described here is a relatively simple procedure, the application of this technique may provide a valuable new tool in a variety of biochemical investigations.

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