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Ability of Tryptophan tRNA to Hybridize with 35S RNA of Avian Myeloblastosis Virus and to Prime Reverse Transcription *In Vitro*

(DNA synthesis/RNA tumor viruses/tRNA as primer)

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ABSTRACT **Selected species of 4S RNA of chick embryo cells will hybridize *in vitro* with 35S RNA of avian myeloblastosis virus. A major tRNA component of the hybridizable 4S RNA is tryptophan tRNA. A hybrid prepared from purified tryptophan tRNA and 35S RNA of avian myeloblastosis virus *in vitro* is an efficient template-primer for DNA synthesis catalyzed by reverse transcriptase (RNA-dependent DNA polymerase).**

Initiation sites or primers are required for reverse transcription of 35S RNA of RNA tumor viruses *in vitro*. It has been reported that in avian myeloblastosis virus (AMV) (1) and in Rous sarcoma virus (2, 3) the primer is a low-molecular-weight RNA. In AMV, 4S RNA with structural (4) and functional (5) properties of transfer RNA has been shown to be closely associated with the viral 70S RNA. A 4S RNA capable of priming DNA synthesis in Rous sarcoma virus has been isolated from the virus and has been shown to be a single species (6, 7). A tRNA with similar properties has been demonstrated in both avian and mammalian cells and was reported to be specifically aminoacylatable with tryptophan (8).

We have recently reported that distinct species of tRNA from a variety of mammalian cells in culture will hybridize with 35S RNA of AKR murine leukemia virus and of AMV *in vitro* (9). We now report that distinct species of 4S RNA of chick embryo cells will hybridize *in vitro* with AMV 35S RNA and that a major component of this RNA is tryptophan tRNA. Furthermore, this tRNA, when hybridized to AMV 35S RNA, will prime the synthesis of DNA in the presence of purified AMV RNA-dependent DNA polymerase.

MATERIALS AND METHODS

The 35S RNA was prepared from AMV-containing chicken plasma (kindly provided by Dr. J. W. Beard of Life Sciences, Inc., Gulfport, Fla.) as described (9). 4S RNA of chick embryo cells, both radioactively labeled and nonlabeled, was prepared from primary cultures. The cells were grown in medium 199 in either petri plates or in roller bottles. ^{32}P labeling was in medium 199 (containing 5% fetal calf serum) with 10% of the usual phosphate content, and RNA extractions were the same as reported (9). RPC-5 column packing was prepared as described (10). Chromatographic conditions were optimized for the separation of eukaryotic tRNAs by C. K. Koh of this laboratory (unpublished results).

RESULTS AND DISCUSSION

4S RNA Components That Hybridize with AMV 35S RNA. When labeled 4S RNA from chick embryo cells is incubated with purified AMV 35S RNA under hybridizing conditions and the 35S RNA reisolated by velocity centrifugation, a fraction of the 4S RNA sediments along with the 35S RNA (Fig. 1). Assuming that the specific activity of the hybridized RNA was uniform, approximately 0.77 μg of 4S RNA hybridized with 27 μg of 35S RNA. The hybridized material was dissociated from the 35S RNA, combined with carrier unlabeled 4S RNA of chick embryo cells (which had been subjected to the same conditions of hybridization but without 35S

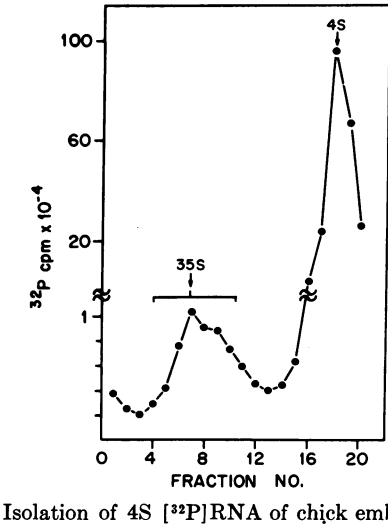

FIG. 1. Isolation of 4S [^{32}P]RNA of chick embryo cells that hybridize with AMV 35S RNA. The hybridization mixture contained 27 μg of AMV 35S RNA and 40 μg of radioactive 4S RNA of chick embryo cells (specific activity = 154,400 cpm/μg) in a solution of 10 mM Tris·HCl (pH 7.6); 0.1 M NaCl; 0.1% sodium dodecyl sulfate; and 1 mM EDTA. The total mixture, 385 μl, was contained in 1-ml ampules, which were flushed with nitrogen before sealing. The contents were heated to 80° for 5 min and quickly chilled in ice water. The sample was then heated at 65° for 30 min, and the temperature of the water bath reset and maintained at 55° for an additional 14–16 hr (approximately 75 min were required for the temperature to reach 55°). After hybridization the samples were quickly chilled in ice water, and the hybridized 4S RNA was separated from the free 4S RNA by sucrose density gradient centrifugation at 50,000 rpm in an SW 56 rotor through a 10–30% sucrose gradient in a solution of 10 mM Tris·HCl (pH 7.6); 10 mM NaCl; 0.1% sodium dodecyl sulfate; and 1 mM EDTA. Fractions were collected from the bottom of the tubes, and ^{32}P was detected directly by measurement of Cerenkov radiation in a liquid scintillation counter (15). In the absence of 35S RNA no radioactivity sediments in the region of fractions 1–13 (see ref. 9). The results shown represent one-half of the hybridization mixture.

Abbreviation: AMV, avian myeloblastosis virus.

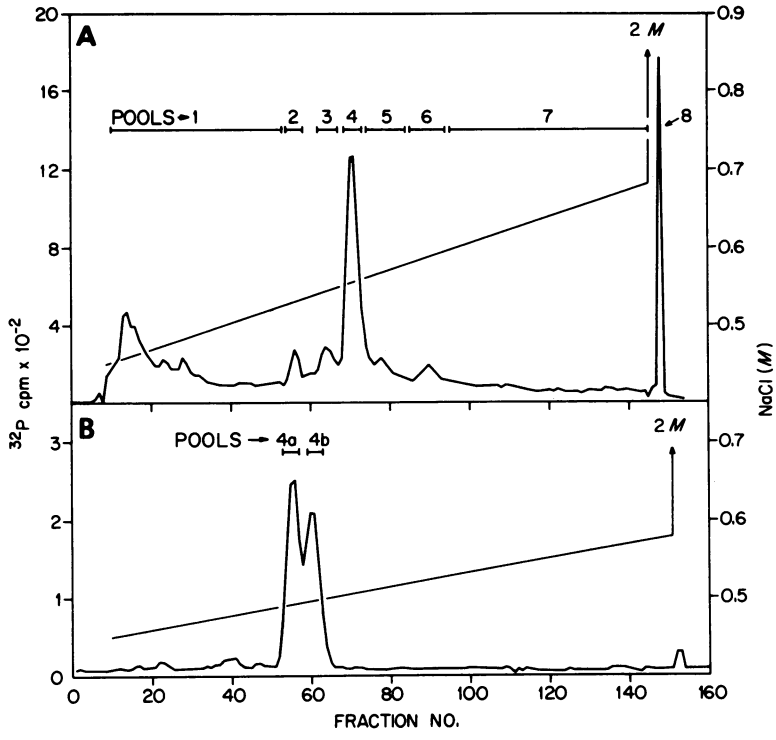

FIG. 2. Separation by RPC-5 chromatography of 4S RNAs that hybridize with AMV 35S RNA. (A) The material illustrated in Fig. 1 that sedimented between fractions 5 and 11 was pooled and collected after precipitation from ethanol. The labeled 4S RNA (37.5% of the total hybrid) was dissociated from 35S RNA by heating to 80° for 5 min and chilling quickly in ice water. 4S RNA was separated from 35S RNA by sucrose density centrifugation. To the labeled 4S RNA was added 425 μg of unlabeled 4S RNA of chick embryo cells, and the mixture was applied to 0.6 × 24 cm RPC-5 column. The column was eluted at 37° with a 200-ml linear gradient from 0.45 to 0.68 M NaCl in 10 mM sodium acetate (pH 4.5), 10 mM $MgCl_2$, 1 mM EDTA, and 5 mM 2-mercaptoethanol. ^{32}P was determined by direct counting of its Cerenkov radiation. (B) The material eluting in fractions 69–73 was pooled and rechromatographed on the same RPC-5 column at 23° using a 0.45–0.58 M NaCl gradient. The indicated regions of the two chromatograms were pooled separately, precipitated from ethanol, and collected by centrifugation and Millipore filtration.

RNA), and fractionated by RPC-5 chromatography. Eighteen to 20% of the hybridized material chromatographed as a single peak eluting at 0.55 M NaCl (Fig. 2A). This result is very similar to that observed when 4S RNA of AKR mouse embryo cells is hybridized with 35S RNA of either AKR murine leukemia virus or AMV (9). Comparison of Fig. 2A with Fig. 5A, which shows the profile of total 4S RNAs of chick embryo cells, demonstrates that the hybridized 4S RNA molecules represent only a select few of the total cellular 4S RNAs separable by this technique. Preliminary fingerprint analyses (kindly performed by Dr. Jack Nichols, Duke University, Durham, N.C.) indicated that the main hybridized component, fractions 69–74 (Fig. 2A), was not a single component. Further resolution into at least two components was achieved by rechromatography at a lower temperature (Fig. 2B). This rechromatography resulted in about an equal split of the hybridizable components, as indicated by ^{32}P, between the fractions indicated 4a and 4b (Fig. 2B). However, the absorbance representing the total cellular RNA added as carrier was not distributed equally between 4a (about 90%) and 4b (about 10%). This further illustrates the nonrandomness by which the cellular 4S RNA associates with the viral 35S RNA.

Capacity of Hybridizable 4S RNAs to Prime DNA Synthesis In Vitro. The ability of 4S RNA molecules to hybridize with viral 35S RNA fulfills an important requirement expected of primer molecules. The relative ability of selected pools of the hybridizable fractions (pools 2, 3, 4a, 4b, 6, and 8) to prime the synthesis of DNA from 35S RNA was then tested after hybridization to AMV 35S RNA. Relative to the unprimed 35S RNA, most of the 4S RNA pools stimulated the synthesis of DNA (Fig. 3). The low amount of synthesis observed with 35S RNA alone and with the hybrid produced from pool 8 was not proportional to RNA concentration, whereas in all the other cases synthesis was proportional to RNA concentration at and below that shown. It is unlikely that the RNA in pool 8 is tRNA (see ref. 9). Pools 3 and 4b were particularly active in priming DNA synthesis. On the basis of the 4S RNA concentration in the hybridization reaction, pool 4b was a particularly efficient primer compared with the other RNA pools tested. 35S RNA primed with pool 4b is 75–80% as effective as native 70S RNA as a template. It is also evident from these results that more than one species of 4S RNA is capable of serving as primer, e.g., pools 2, 3, and 6 also show priming activity. Without knowing which tRNAs these pools contain and their relative purity, it is difficult to properly compare their absolute priming effectiveness.

Tryptophan tRNA as a Major Component of the Hybridizable 4S RNA. Because of their elution position from RPC-5 and because of the base composition of similar hybridized 4S RNAs in other systems (9), it is likely that the RNAs in pools 2 to 6 (Fig. 2A) are tRNA. It is not uncommon for aminoacylated tRNAs to chromatograph differently from the nonacylated form in the RPC systems (ref. 12 and unpub-

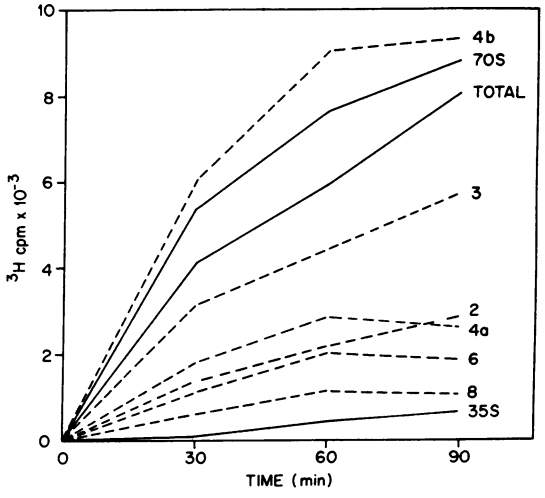

FIG. 3. The relative capacity of the 4S RNA fractions of chick embryo cells that hybridize with AMV 35S RNA *in vitro* to prime DNA synthesis. RNA in the indicated pools from Fig. 2 was hybridized in a total volume of 285 μl with 7 μg of AMV 35S RNA, as indicated in Fig. 1. The approximate amounts of 4S RNA in the pools that were hybridized were: pool 2, 23 μg; 3, 11 μg; 4a, 5 μg; 4b, 0.55 μg; 6, 61 μg; and 8, 12 μg. Each hybridization reaction was subjected to sucrose density gradient centrifugation (without sodium dodecyl sulfate), and the hybrid region was pooled, precipitated from ethanol, and collected into 200 μl of 10 mM Tris·HCl (pH 7.6); 10 mM NaCl; and 1 mM EDTA. Twenty-microliter aliquots were assayed with purified AMV reverse transcriptase (11). Assuming complete recovery, the amount of hybridized 35S RNA used in each assay is estimated to be 0.7 μg. The amount of 35S RNA (lower curve) used was 0.5 μg; 70S RNA was 0.5 μg and the "total" hybrid, as shown in Fig. 1, was estimated to be 0.68 μg. The assay mixture contained in a total volume of 50 μl: RNA as indicated; 5 μl of AMV reverse transcriptase, which when $(A)_n \cdot (dT)_n$ was used as a template catalyzes the incorporation into DNA of 580 pmol of dTMP per hr; 0.5 mM $MgCl_2$; 0.5 mM $MnCl_2$; 10 mM dithiothreitol; 0.17 mg/ml of bovine serum albumin; 128 mM KCl; 10 μM each of [^{3}H]dATP, [^{3}H]dTTP, [^{3}H]dCTP, and [^{3}H]-dGTP, specific activity 8.48 Ci/mmol; 20 mM Tris·HCl (pH 8.0). Fifteen-microliter aliquots were taken at the times indicated, and DNA synthesis was determined by described methods (11).

lished observations). Preliminary experiments showed that the hybridizable 4S RNA prepared with radioactively labeled 4S RNA of AKR mouse embryo cells and AMV 35S RNA, when aminoacylated with a mixture of basic amino acids (tryptophan, lysine, histidine, and arginine), had a chromatographic profile markedly different from that observed before aminoacylation. Significant amounts of the hybridizable material then eluted coincident with the major lysine isoacceptor species and also in the position of the two tryptophyl-tRNAs seen in the mouse cell tRNA. On this basis and considering the likelihood that "spot 1" RNA described by Sawyer *et al.* (8) is tryptophan tRNA, 4S RNA of chicken embryo cells, hybridized to and then dissociated from AMV 35S RNA, was aminoacylated either with lysine alone or with both lysine and tryptophan; the resulting products were compared by RPC-5 chromatography with a nonacylated sample. The main component of the nonacylated hybridizable 4S RNA elutes just after the major lysine isoacceptor at approximately 0.55 M NaCl (Fig. 4A). Aminoacylating the hybridizable 4S RNA with lysine appears to cause the shift of some of the radioactivity from the main peak (fractions 53–57) to the position of the major lysine isoacceptor at fractions 48–52 (Fig. 4B). In marked contrast to the results shown in Fig. 4A and B, aminoacylation with tryptophan causes a pronounced shift in the RPC-5 profile of the hybridizable 4S RNA. A large amount of the hybridizable material that eluted at 0.55 M NaCl (Fig. 4A and B) now elutes at 0.6 M NaCl coincident with the main tryptophyl-tRNA of the chick embryo cells (Fig. 4C).

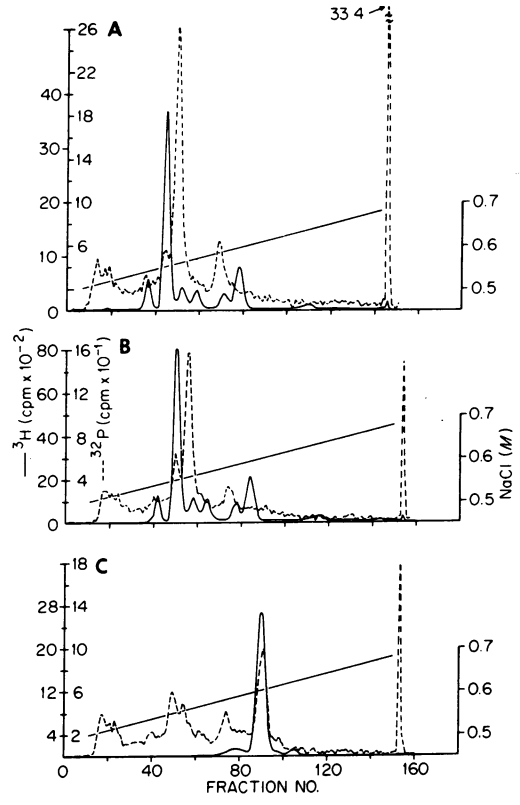

FIG. 4. RPC-5 chromatography of ^{32}P-labeled 4S RNA of chick embryo cells that hybridize with AMV 35S RNA: (A) nonaminoacylated and chromatographed along with [^{3}H]lysyl-tRNA of chick embryo cells; (B) aminoacylated with [^{3}H]lysine; (C) or aminoacylated with nonlabeled lysine and [^{3}H]tryptophan. Hybridizable 4S RNA was prepared and isolated as described in the legends of Figs. 1 and 2 starting with 16 μg of chick cell 4S [^{32}P]RNA (74,280 cpm/μg) and 15 μg of AMV 35S RNA. Nonlabeled 4S RNA of chick embryo cells (450 μg) was added to the dissociated hybridizable 4S RNA, and the mixture was collected by ethanol precipitation. Aminoacyl-tRNA synthetases were prepared and the aminoacylations were carried out under conditions described (13). The specific radioactivity of the lysine used was 3,390 mCi/mmol and that of the tryptophan was 4,500 mCi/mmol. Chromatography was as described in the legend of Fig. 2. The fractions were precipitated with acid in the presence of carrier calf thymus DNA (100 μg per fraction) and collected on Whatman GFC glass fiber disks. Radioactivity was determined by liquid scintillation counting.

Since the radioactivity shifts all seem to be at the expense of that observed as the major peak in the nonacylated sample (fractions 47–55 of Fig. 4A; the peak in fractions 70–80, for example, does not appear to change as a result of aminoacylation), a rough estimate of the amount of aminoacylatable tryptophan and lysine tRNAs in that peak can be made.

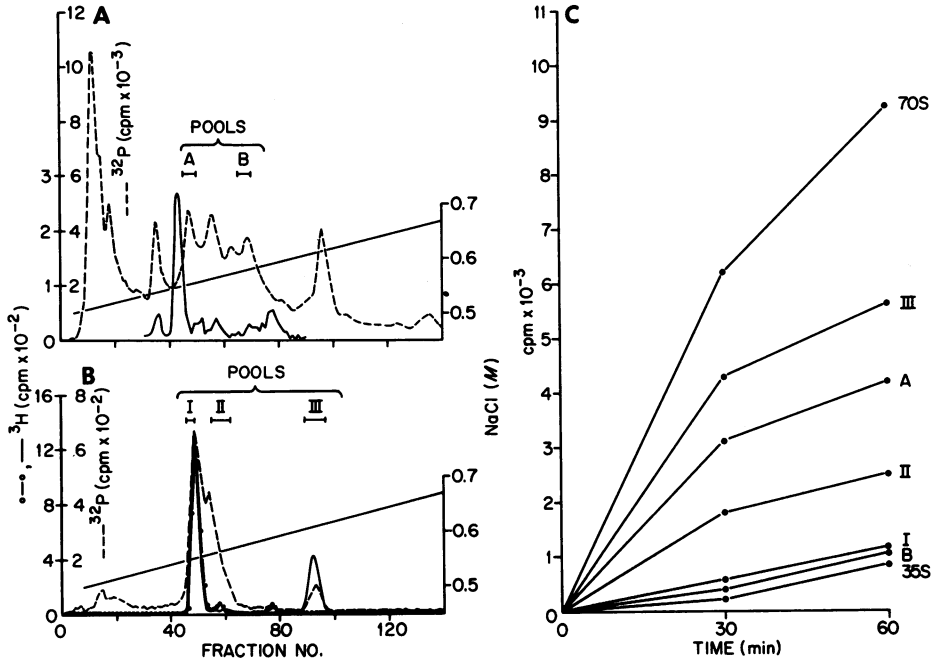

FIG. 5. Isolation of tryptophan tRNA and its ability to prime the synthesis of DNA when hybridized to AMV 35S RNA. (A) RPC-5 chromatography of 650 μg of ^{32}P-labeled 4S RNA of chick embryo cells (356 cpm/μg), of which 80 μg was aminoacylated with [^{3}H]lysine (3, 390 mCi/mmol). ^{32}P was determined directly by counting its Cerenkov radiation. Tritium was detected by counting 0.1-ml aliquots in an aqueous scintillation counting system. Pools A and B were made as indicated. (B) The equivalent of two-thirds of pool A was aminoacylated with [^{3}H]lysine (3, 390 mCi/mmol) and [^{3}H]tryptophan (4, 500 mCi/mmol) and rechromatographed on RPC-5. Radioactivity was determined as in panel A. *Open circles* represent an identical chromatogram in which 10% of pool A was aminoacylated with [^{3}H]lysine only. In this case the total fraction was counted on glass fiber disks as described in the legend of Fig. 4. (C) The RNAs in the pools indicated in panels A and B were collected and tested for their ability to prime the synthesis of DNA when hybridized to AMV 35S RNA. Each pool—A, 12.9 μg; B, 11.6 μg; I, 3.6 μg; II, 4.2 μg; and III, 1.0 μg—was hybridized with 3.4 μg of AMV 35S RNA in a total volume of 200 μl. Other conditions for hybridization and for collecting the hybrids were as described in the legends of Figs. 1 and 3. Twenty-microliter aliquots of each were assayed with purified AMV reverse transcriptase (11), as described in the legend of Fig. 3. Assuming complete recovery, the amount of the "pool" hybrids assayed is estimated to be 0.34 μg of 35S RNA. The curve labeled 35S also represents an estimated 0.34 μg that had been through the same hybridization conditions as the pools. 70S RNA (0.25 μg) was assayed. Twenty-microliter aliquots were taken at the times indicated, and DNA synthesis was determined by methods described (11).

Approximately 58% of the peak is shifted to the region of tryptophyl-tRNA by aminoacylating with tryptophan, 17% to the lysyl-tRNA region by aminoacylating with lysine, and 25% remains at the original position. It is not known whether the 25% remaining is due to incomplete aminoacylation with tryptophan and lysine or whether it represents a still unidentified component.

Tryptophan tRNA as a Primer for DNA Synthesis. The difference in chromatographic property of nonacylated and acylated tryptophan tRNA is remarkable and probably indicates a structural change more complex than just the addition of the amino acid. This property of tryptophan tRNA provides a convenient and specific method for the purification of tryptophan tRNA. To do this and to determine whether tryptophan tRNA would specifically prime the synthesis of DNA using AMV 35S RNA, the experiment illustrated in Fig. 5 was done. ^{32}P-Labeled 4S RNA of chick embryo cells was chromatographed with marker RNA aminoacylated with [^{3}H]lysine (Fig. 5A). Based on the data shown in Fig. 4A, the material included in pool A should contain the major 4S RNA components that hybridize with AMV 35S RNA. Also, since the main portion of the RNA applied to the column was not aminoacylated (except for the approximate 12% aminoacylated with lysine), pool A contained tryptophan tRNA and lysine tRNA. Pool B was chosen arbitrarily as a control to be used in priming assays. Pools A and B each contained approximately 60 μg of RNA. Aminoacylation of two-thirds of pool A with [^{3}H]lysine and [^{3}H]tryptophan and rechromatography on RPC-5 gave the results shown in Fig. 5B. In the region of fractions 46–64 the RNA, represented by ^{32}P, is resolved into two main components, the first of which is coincident with the major peak of tritium. An additional amount of RNA (about 9%) peaks in fractions 89–96 and is again coincident with a peak of tritium. Chromatography of a small fraction (about 10%) of pool A aminoacylated with [^{3}H]lysine alone showed that the tritium seen in fractions 46–64 is lysyl-tRNA and, therefore, the tritium at fractions 89–96 is tryptophyl-tRNA. It is interesting that tryptophan tRNA comprised about 9% of pool A (Fig. 5A) and that, likewise, the RNA concentration of pool 4b in Fig. 2B was about 10% of pool 4 shown in Fig. 2A. It is likely that pool 4b (Fig. 2B) is tryptophan tRNA. These data (Fig. 5A and B) also indicate that nonacylated lysine tRNA and tryptophan tRNA chromatograph in the same position on RPC-5. Aminoacylation of lysine tRNA also changes its elution property on RPC-5 (Fig. 5B), and unlike tryptophan tRNA, to a lower salt concentration. It is the major lysine isoacceptor tRNA that coelutes, nonacylated, with nonacylated tryptophan tRNA (unpublished results).

With lysyl-tRNA as a marker, a fraction of total cellular RNA corresponding to the major 4S RNA components that hybridize to AMV 35S RNA can be isolated (Fig. 5A). Specific aminoacylation with lysine and tryptophan followed by rechromatography resulted in further separation of this fraction into partially purified lysine tRNA (pool I, Fig. 5B), an unidentified component (pool II, Fig. 5B), and, at least theoretically, pure tryptophan tRNA (pool III, Fig. 5B). Fig. 5C illustrates the relative ability of the RNA in the pools indicated in Fig. 5A and B to prime the synthesis of DNA from AMV 35S RNA. 35S RNA alone and hybrids formed from pools B and I are inactive. Pools A, II, and III are all effective primers for DNA synthesis. As in the case of pool 4b (Fig. 2B), pool III is, on a weight basis especially, a very effective primer. Probably a major portion of the priming activity of pool A is due to pool III; however, the activity in pool II might suggest that another uncharacterized primer is present. Alternatively, the activity in pool II might indicate incomplete aminoacylation of pool A with tryptophan and reflect residual amounts of tryptophan tRNA in this pool. It is interesting that pool I, which should be considerably enriched in lysine tRNA, is inactive, especially in view of the fact that a detectable portion of the 4S RNA of chick embryo cells that hybridizes with AMV 35S RNA appears to be lysine tRNA (Fig. 4B).

It appears from the data presented that tryptophan tRNA is a major component of the chick embryo cell tRNA that hybridizes with AMV 35S RNA *in vitro*. Tryptophan tRNA purified based on a method using its different chromatographic property in the nonacylated in contrast with the acylated state is a particularly effective primer for DNA synthesis with AMV 35S RNA as the template. Using the same technique as illustrated in Fig. 5 we have obtained tryptophan tRNA from chicken liver with a specific tryptophan acceptor activity of 1438 pmol/50 μg of RNA. This preparation is very effective as a primer for DNA synthesis. Purified tryptophan tRNA alone does not support DNA synthesis. Furthermore, an active hybrid prepared with purified tryptophan tRNA and AMV 35S RNA is rendered inactive by heating to 80° for 3–5 min followed by quick cooling. The association of the primer with 35S RNA at 55° and its dissociation at 80° are results that are consistent with previously reported properties of the Rous sarcoma virus primer (2).

Elder and Smith (14) reported that the tRNA that can be dissociated from AMV 70S RNA is enriched in methionine tRNA 1. They suggested that methionine tRNA 1, with its many unique properties and its close association with viral 70S RNA, might act as a primer. We have no evidence that methionine tRNA of chick embryo cells will hybridize *in vitro* with AMV 35S RNA, although the region of the gradient indicated by the combined fractions included in pools 2 and 3 (Fig. 2A) is the position of methionine tRNA 1 (numbered as in ref. 14). Furthermore, chicken liver methionine tRNA 1 at a purity of 300 pmol/50 μg of RNA is not an effective primer in our assay system. Presently we are attempting to further purify methionine tRNA 1 in order to critically assess its potential to prime DNA synthesis using AMV 35S RNA *in vitro*.

Whether these interactions that have been demonstrated *in vitro* are relevant to the situation *in vivo* is not known. These data do, however, suggest a potential role for specific tRNAs in the replication of RNA tumor viruses. We are currently applying these same techniques to the study of other viral RNA and cellular RNA interactions. Preliminary results indicate that the specificity of the mouse cellular tRNAs that hybridize with murine tumor virus RNA is different from that shown with chick embryo cell tRNA and AMV 35S RNA.

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