IN VITRO ASSOCIATION OF UNIQUE SPECIES OF CELLULAR 4S RNA WITH 35S RNA OF RNA TUMOR VIRUSES*

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SUMMARY — Unique 4S RNA species from AKR mouse embryo cells hybridize with AKR murine leukemia virus and avian myeloblastosis virus 35S RNAs in vitro. Analyses by reversed-phase column chromatography indicate that the major 4S species that hybridize with the two viral RNAs are probably the same. A 4S RNA species with similar chromatographic properties is a major component of the AKR viral 4S RNA which associates with the viral 70S RNA in vivo.

Low molecular weight RNAs (4-8S), both in a "free" form (1,2) and in association with the 70S RNA component (3), have been shown to be within RNA tumor viruses. Transfer RNA is the major component of both "free" and 70S-associated 4S RNAs (3-6). Various experimental approaches have all implicated 4S RNAs as primers for reverse transcription of viral RNA to DNA (7-9). Recently Dahlberg, et al. (10) reported the isolation and identification of a 4S RNA primer in Rous sarcoma virus. Its nucleotide composition and oligonucleotide fingerprint were similar to that of tRNA (11).

Most of the studies to date concerning the structure and function of the low molecular weight RNAs of RNA tumor viruses have been done with avian viruses. Less is known about the murine viruses (12), since relatively low virus quantities are obtainable with existing murine systems. In a series of experiments studying 4S RNAs of RNA tumor viruses (13 and manuscript in

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preparation), we have found that the AKR murine leukemia virus is similar to avian sarcoma virus with respect to heat-dissociation of 4S RNA from 70S RNA (7). This would suggest that extensive base complementarity exists between the 70S-associated 4S RNAs, including the primer molecule, and the viral 35S or 70S RNA. In this communication we report results of hybridization experiments demonstrating association of specific cellular 4S RNA species with 35S or 70S RNAs of RNA tumor viruses.

METHODS

Viral RNA Preparation — AKR virus was produced in culture from spontaneously activated high passage AKR mouse embryo cells. Eagle's minimal medium containing Earle's salts supplemented with 10% fetal calf serum was used. Hydrocortisone at a final concentration of 10^{-6} M was added to the medium when the cells reached confluency. Hydrocortisone slightly enhances virus production and helps prevent cell detachment. Cell debris was removed from culture medium collected at four hr intervals by centrifugation at 8,000 RPM for 10 min in a type SS-34 Sorvall rotor. Virus was then pelleted through 20% sucrose (in TNE - 0.01 M Tris·HCl, pH 7.6; 0.1 M NaCl and 0.001 M EDTA) by centrifugation at 25,000 RPM for one hr in a SW27 Spinco rotor. Pelleted virus was resuspended in TNE and isopycnically banded in a 20-60% sucrose in TNE gradient (12-16 hrs at 25,000 RPM in a SW27 rotor). The banded virus was again concentrated by pelleting and resuspended in 0.05 M Tris·HCl, pH 9.0; 0.1 M NaCl; 0.1% SDS and 0.001 M EDTA. The turbid solution was clarified by the addition of 0.2 vol of 5% SDS and the sample extracted 2-4 times with pH 9.0 buffer-saturated phenol containing, per ml, 0.123 ml m-cresol and 1.12 mg 8-hydroxyquinoline. The appropriate RNA component was obtained by separation on 10-30% sucrose density gradients in 0.01 M Tris.HCl, pH 7.6; 0.01 M NaCl; 0.05% SDS and 0.001 M EDTA (TNSE). Chicken plasma containing AMV was obtained from Dr. J. W. Beard of Life Sciences, Inc. Avian myeloblastosis virus (AMV) RNA was prepared as described for AKR.

Cellular 4S RNA Preparation — Cells were grown in 20 X 100 mm petri plates in Eagle's minimal medium containing Earle's salts and 10% fetal calf serum. Prior to confluency, the actively dividing cells were labeled for 48 to 72 hrs (radioactive medium was changed every 24 hrs) with [^{3}H]-uridine, 50 μC/ml (specific activity of 29 to 45 C/nmole) or carrier free [^{32}P] phosphate, 50 μC/ml (phosphate labeling was done in medium containing 10% the usual amount of phosphate). The cells were then rinsed on the plates with cold saline, scraped from the plates, suspended in 0.01 M Tris·HCl, pH 7.6; 0.15 M NaCl; 0.005 M $MgCl_2$; 0.001 M EDTA and extracted 3-4 times with phenol containing m-cresol and 8-hydroxyquinoline. The 4S RNA was further purified by precipitating the bulk of the rRNA with 1 M NaCl followed by chromatography on DEAE-cellulose (14). Final traces of rRNA were removed by sucrose density gradient centrifugation. Specific activities in the range of 100,000 to 600,000 cpm/μg RNA are obtainable by this method.

Hybridization — A typical hybridization reaction (80-150 μl) consisted of 2 to 10 μg of viral 35S or 70S RNA and radioactive cellular 4S RNA at a weight ratio of 1 to 5 times the viral RNA in a solution containing 0.01 M Tris·HCl, pH 7.6; 0.1 M NaCL; 0.1% SDS and 0.001 M EDTA. The 4S RNA was heated to 80° for 15 min, quick-cooled and added to the complete reaction mixture contained in 1 ml ampules which were then flushed with nitrogen and sealed. The samples were heated to 65° for 30 min in a water bath and then the temperature was set at 55° and held for 16 hrs (under the conditions used, the temperature dropped to 55° in 75 min). After the reaction, the samples were quick-cooled in ice water and the 4S RNA associated with viral 35S RNA was separated from bulk 4S RNA directly by velocity sedimentation in 10-30% sucrose gradients in TNSE.

Analyses of 35S Associated 4S RNA — Radioactivity sedimenting in the 35S RNA region of the gradient was pooled, heated to 80° for 3 min and quick-cooled, adsorbed to and eluted from DEAE-cellulose and finally analyzed by RPC-5 column chromatography (15). SDS polyacrylamide gel

electrophoresis in 10% gels was done according to the method of Loening (16). Base compositions were determined by anion exchange chromatography (17).

RESULTS

Sucrose gradient analysis shows that in the presence of AKR viral 70S RNA, under the hybridization conditions described, a small amount of the tritiated AKR cell 4S RNA sediments in the 35S and higher region of the gradient (Figure 1). In the absence of viral RNA no radioactivity appears in this region. Essentially identical results are obtained using AKR 35S

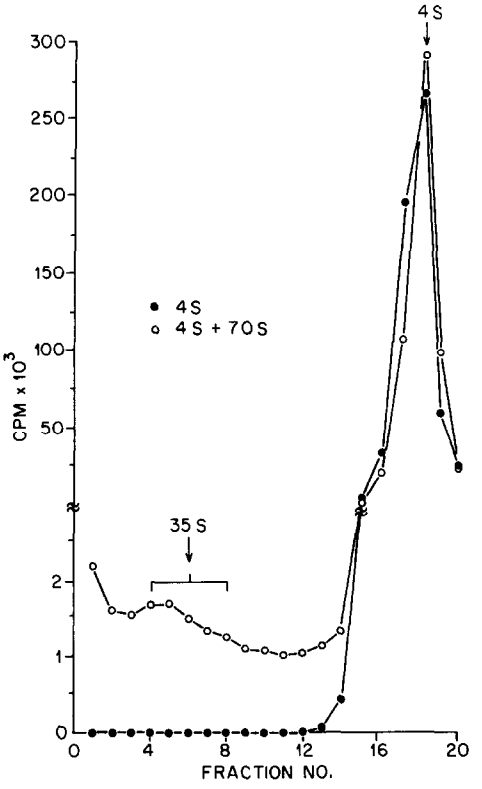

FIG. 1. Analysis by sedimentation in 10-30% sucrose gradients of hybridization reactions containing AKR 70S RNA, 9.2 µg, and [^{3}H]uridine labeled AKR cell 4S, 10.2 µg or 4S RNA alone. Sedimentation was in the SW56 Spinco rotor at 50,000 RPM for 4 hours. Fractions were obtained from the bottom of the tube and the radioactivity of approximately 10% (20 λ) of each fraction was measured.

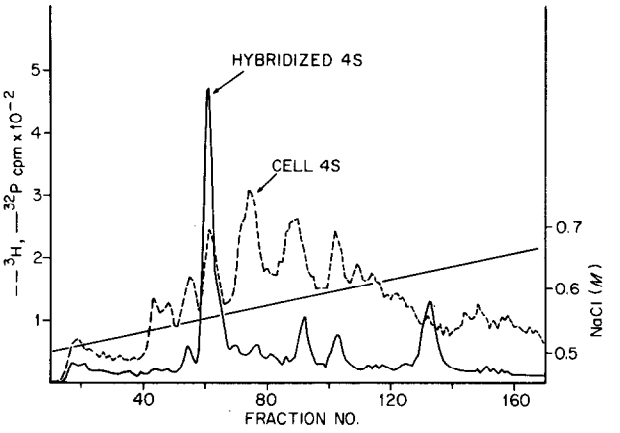

FIG. 2. RPC-5 co-chromatography of [^{32}P]labeled AKR cell 4S RNA hybridized to AKR 35S RNA with [^{3}H]uridine labeled AKR cell 4S RNA. The gradient was 200 ml, 0.5-0.68 M NaCl, and the chromatography was done at 37°.

viral RNA, except that the radioactivity peaks much more sharply in the 35S region of the gradient. Although the conditions of hybridization should completely dissociate 70S RNA to 35S, the increase in radioactivity seen at the bottom of the gradient (fractions 1 and 2, Figure 1) is a consistent observation with 70S viral RNA but not with isolated 35S RNA.

RPC-5 analysis of the 4S RNA which associates with viral 35S RNA shows it to be distinctly different from the total cellular 4S RNA (Figure 2). The 4S RNA, which associates with 35S RNA and elutes within the salt gradient used, chromatographs mainly as one component (fractions 59-65, Figure 2). However, the minor peaks, particularly that at fraction 133, are consistently observed. Figure 3 shows a comparison of the 4S RNA which associates with 35S viral RNA *in vitro* with the 4S RNA obtained by heat dissociation of 70S RNA (70S-associated 4S) from [^{32}P]labeled AKR virus. A major RNA component associated with 70S viral RNA *in vivo* co-chromatographs with the main component which associates with viral 35S RNA *in vitro* (fraction 59, Figure 3). Efforts to characterize these two components in order to establish their identity or nonidentity are in progress.

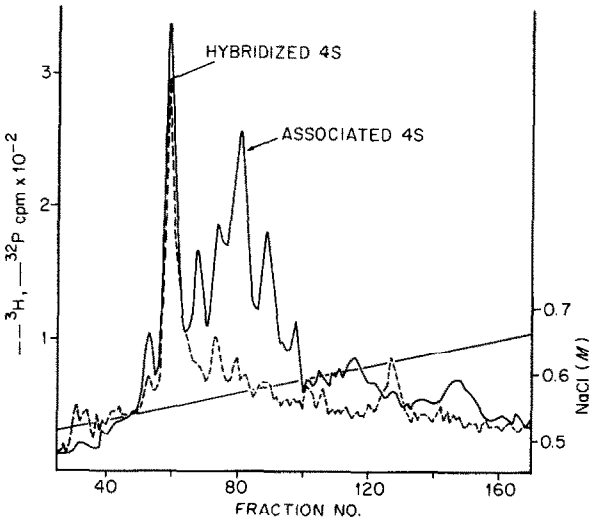

FIG. 3. RPC-5 co-chromatography of [^{3}H]uridine labeled AKR cell 4S RNA hybridized to AKR 35S RNA with [^{32}P]labeled 4S RNA obtained from *in vivo* labeled AKR viral 70S RNA by thermal dissociation.

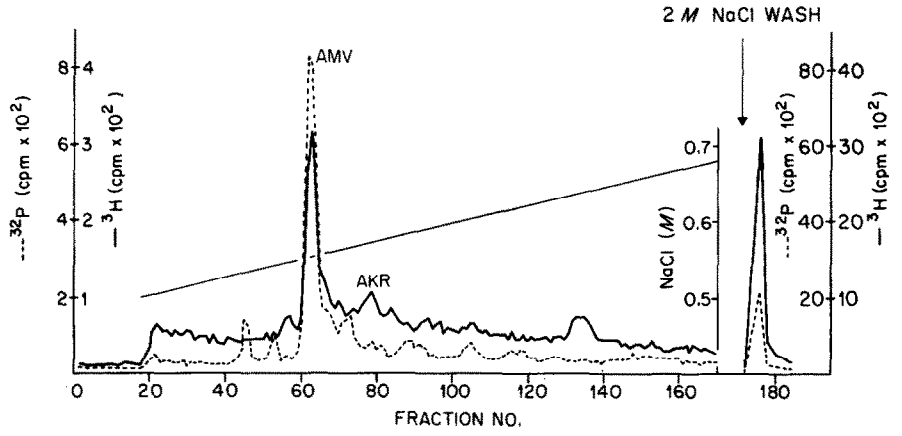

FIG. 4. RPC-5 co-chromatography of [^{3}H]uridine labeled AKR cell 4S RNA hybridized to AKR 35S RNA with [^{32}P]labeled AKR cell 4S RNA hybridized to AMV 35S RNA.

AKR cellular 4S RNA also associates *in vitro* with AMV 35S RNA, the main component of which co-chromatographs with that which associates with AKR 35S RNA (Figure 4, fractions 60-65). The minor components are generally not coincident. It can be seen also in Figure 4 that a significant amount of RNA associating with both AKR and AMV 35S RNAs does not elute from

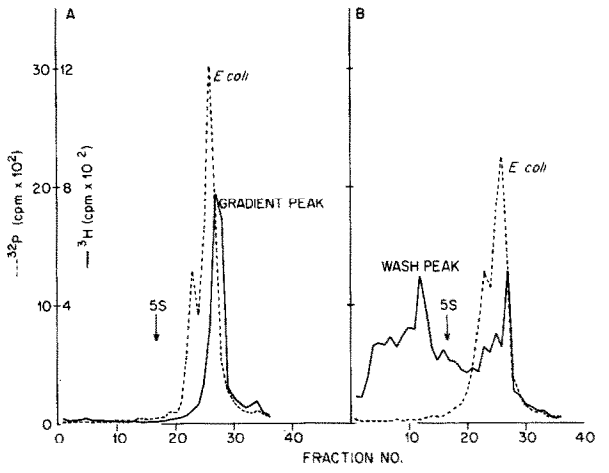

FIG. 5. Comparison by SDS polyacrylamide gel electrophoresis of the two major components from RPC-5 of [^{3}H]uridine labeled AKR cell 4S RNA which hybridized to AKR 35S RNA in vitro. (A) Major RNA component eluting within the gradient. (B) RNA eluted with 2 M NaCl. [^{32}P]labeled E. coli 4S RNA is used as a marker.

RPC-5 in the region of most 4S RNA, but requires a higher NaCl concentration for elution. This RNA comprises at least 20% of the total material recovered from the column. The amount of material eluting in the wash (wash peak) from RPC-5 appears to increase as the quality of the input 4S RNA used for hybridization decreases.

The electrophoretic mobilities of the main peak eluting from RPC-5 within the gradient (fractions 60-64, Figure 4) and the wash peak are compared in Figure 5. The gradient peak migrates with the light side of marker E. coli 4S RNA. The material from the wash is heterogeneous in size, ranging from 4S to 7S. Table I shows the major nucleotide composition of the same two fractions. The gradient peak RNA has a composition similar but not identical to that of the total cellular 4S RNA (input RNA). Minor nucleotides characteristic of tRNA are also in this fraction. In contrast the material eluted in the wash is distinctly different from that of the input RNA and is characterized by a high A + U content. These data, together with the position of elution from RPC-5, suggest that the bulk

TABLE 1

Nucleotide composition of hybridized RNA

RNA	Percentage as: CMP	UMP	AMP	GMP	$\frac{A+U}{G+C}$
Gradient peak	33.6	14.5	20.5	31.4	0.54
Wash peak	23.2	27.3	27.6	22.0	1.22
Cellular 4S RNA (input)	33.9	22.1	19.0	25.1	0.70

of the material eluting from RPC-5 in the wash is probably degraded messenger RNA.

DISCUSSION

Studies done with the 4S RNA primer of Rous sarcoma virus (7,9,10) indicate extensive base complementarity between it and the viral 70S or 35S RNA. We have shown that unique 4S RNA species from AKR mouse embryo cells associate with AKR and AMV 35S RNAs *in vitro*. RPC-5 chromatographic analysis reveals one major component which elutes within the tRNA region of the salt gradient. This component associates to the extent of at least 3 copies per 70S and has several of the properties of tRNA. A similar, if not identical, component has been demonstrated in AKR low passage (non virus producing) mouse embryo cells, Balb/c mouse embryo cells as well as a human rhabdomyosarcoma cell line, RD (unpublished data). Oligonucleotide fingerprinting should establish the relationship between this component which associates with viral 35S RNA *in vitro* and the "spot 1 RNA" of Rous sarcoma virus (10,18) that has been recently demonstrated in a variety of cells (19).

Results of our attempts to show primer activity with the *in vitro* associated 4S RNA have thus far been inconclusive. The relatively poor activity of murine viral DNA polymerases with their natural template-primer

in a reconstituted system (20) indicates that the murine systems might be more complex than the avian systems. The use of large amounts of viral 35S RNA, which can be recycled, should allow us to prepare the in vitro 35S RNA associated 4S RNA in sufficient quantity to determine whether it is structurally and/or functionally important in the replication of RNA tumor viruses.

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