Isolation and partial purification of the major abundant class rat seminal vesicle poly(A⁺)-messenger RNA

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ABSTRACT

Total poly(A⁺)-RNA (poly(A⁺)-RNA_{tot}) was isolated from rat seminal vesicle and its size distribution determined by 70% formamide 5-25% sucrose density analysis. One major peak was resolved in the 10-13 S region and accounted for ~35% of the total poly(A⁺)-RNA applied. Preparative 1% SDS, 5-20% linear sucrose density gradients also resolved a single major peak in the 11S region (poly(A⁺)_{11S}). Analysis of poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} under denaturing conditions on 2% agarose gel electrophoresis demonstrated two major components in both poly(A⁺)-RNA populations. Size estimations for these components are 620 and 540 NT respectively. ³H-cDNA was made to both poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S}. Back-hybridization of poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} to their respective ³H-cDNA revealed a highly abundant class representing 41% and 85% of the sequences in their respective ³H-cDNA's. The highly abundant class corresponded to 3-5 sequences present in 30,000-50,000 copies/cell. *In vitro* translation of poly(A⁺)-RNA_{11S} resulted in two major polypeptides coded for by the 620 NT long and 540 NT long poly(A⁺)-RNA respectively.

INTRODUCTION

RNA/DNA hybridization as introduced by Bishop *et al.* (1) has become a useful tool to determine the amount of genetic information expressed as m-RNA. Such studies have been made in chick oviducts to establish the effect of estrogen on the synthesis of egg white proteins (2,3,4,22,23,24). A class of highly abundant RNA sequences was present in laying hens and estrogen treated chicks, but absent or significantly reduced in chicks withdrawn from hormones. The abundant class of RNA was found to code for egg white proteins. Similar experiments performed with poly(A⁺)-RNA from rat ventral prostate have shown the presence of an androgen dependent abundant class of RNA sequences coding for three major proteins or polypeptides (5). In rat seminal vesicle it has been shown that there is a small set of polypeptides representing 30-50% of the proteins synthesized by the vesicle (7,8,9,15). Poly(A⁺)-RNA from rat seminal vesicle has been investigated (16) using c-DNA

analysis and the result indicate the presence of a highly abundant class of poly(A⁺)-RNA. This report demonstrates the isolation, partial purification and translation of this abundant class of poly(A⁺)-RNA sequences from rat seminal vesicles.

ANIMALS

Adult male CD rats (200-250g) from this institute were used.

MATERIALS

Liquefied phenol was purchased from Fisher Scientific Co. and redistilled before use. Redistilled phenol was equilibrated to 10 mM Tris/HCl, pH 8.0; 100 mM NaCl; 10 mM EDTA and adjusted to 0.1% (w/v) 8-Hydroxyquinoline.

Formamide was purchased from Aldrich Chemical Co. and deionized before use by stirring with Bio-Rad RG 501-X8 mixed bed resin.

SDS, Agarose, and ethidium bromide, electrophoresis grade, were purchased from Bio-Rad Laboratories.

Oligo dT₁₂₋₁₈ (T3) cellulose was purchased from Collaborative Research, Inc.

Proteinase K was purchased from E. M. Laboratories.

Sucrose, ribonuclease-free density gradient grade, was purchased from Schwarz-Mann.

METHODS

RNA Extraction

Rats were sacrificed by decapitation. Seminal vesicles were quickly removed and freed of coagulating glands and connective tissue. As far as possible, secretory fluids were manually expressed, and the seminal vesicles were then frozen in liquid N₂.

Frozen tissue was homogenized in 10 volumes (v/w) of phenol-chloroform (1:1, v/v) plus 10 volumes of a buffer containing 0.075 M NaCl; 0.025 M EDTA, pH 8.3, and 1% (w/v) sodium dodecyl sulfate (SDS) using a semi-micro Waring blender at 20,000 rpm for 60 seconds. The homogenate was immediately shaken 5' with an additional 10 volumes (v/w) of chloroform to disperse the thick emulsion formed. Phases were separated by centrifugation at 10,000 xg for 10'. The aqueous phase was extracted twice more, once with an equal volume of phenol-chloroform (1:1, v/v) and again with an equal volume of chloroform alone. The aqueous phase was adjusted to 0.5 M NaCl and the nucleic acids

were precipitated overnight with 2 volumes 95% Ethanol at -20°C . Precipitates were collected by centrifugation at 10,000 xg for 20' and were redissolved in 10 mM Tris HCl, pH 7.6, 5 mM EDTA, 1% SDS to a concentration of 2-3 mg/ml. Proteinase K was added to a final concentration of 50 $\mu\text{g/ml}$ and the mixture was incubated 30' at 37°C . Following incubation, the mixture was extracted for three times with 1.5 volumes (v/v) of chloroform. The aqueous phase was adjusted to 0.5 M NaCl and nucleic acids were precipitated with 2 volumes of ethanol overnight at -20°C . Precipitates were collected by centrifugation at 10,000 xg for 20' and were dissolved in 10 mM Tris HCl, pH 7.6, 5 mM EDTA, 1% SDS to a concentration ≤ 1 mg/ml. Poly(A⁺)-RNA was separated from the total nucleic acid extract by affinity chromatography, as follows. Total nucleic acid extract in 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, plus 1% SDS was adjusted to 0.5 M NaCl and applied to a column containing ~ 10 g of oligo dT₁₂₋₁₈ cellulose (dT). dT-Bound material was eluted from the column with 10 mM Tris/HCl, pH 7.6, 5mM EDTA, plus 1% SDS. Fractions showing absorbance at 254 nm were combined, diluted five-fold with low salt elution buffer, and heat denatured for 10' at 68°C . Salt was then readjusted to 0.5M NaCl and the diluted sample was applied to the oligo-dT cellulose column a second time at a flow rate of ~ 3.5 ml/minute. Twice dT bound material was eluted from the column with low salt elution buffer. Poly(A⁺)-RNA fractions were combined, adjusted to 0.5 M NaCl, and precipitated overnight with 2 volumes of ethanol.

SDS Sucrose Gradients

11.0 ml Linear 5-20% sucrose density gradients were constructed from stock solutions containing 5% or 20% sucrose in 10 mM Tris/HCl, pH 7.6; 5 mM EDTA, plus 1% SDS using a Buchler Gradient Generator. Poly(A⁺)-RNA samples and rRNA standards ~ 1 mg/ml in deionized water were diluted 1:1 (v/v) with 10 mM Tris/HCl pH 7.6, 5 mM EDTA, plus 1% SDS, and heat denatured for 5 min. at 65°C before being layered onto the gradients. SDS-sucrose gradients were centrifuged at 176,000 xg for 22 hours at 20°C in a SW 41 rotor.

Formamide-Sucrose Gradients

11.0 ml Linear 5-25% sucrose density gradients were prepared from stock solutions containing 5% or 25% sucrose in 3 mM Tris/HCl, pH 7.5, 2 mM EDTA, plus 70% (v/v) deionized formamide. Poly(A⁺)-RNA samples and rRNA standards in 2 mM EDTA plus 70% formamide were heated 1 min at 37°C before being layered onto gradients. Formamide-sucrose gradients were centrifuged at 176,000 xg for 54 hours at 20°C .

Complementary DNA

Synthesis of complementary DNA (cDNA) with AMV reverse transcriptase and

RNA excess hybridizations have been described in detail elsewhere (10).

Alkaline sucrose gradients were performed as described previously (10).

Cell-Free Protein Synthesis

RNA samples were translated in a wheat-germ system (26) containing 1- $[^{35}\text{S}]$ -methionine (980 Ci/mmol, Amersham). Reaction volumes were 50 μl and after 1 h of incubation at 25°C ribosomes were removed by centrifugation at 160,000 $\times g$ for 15 min. in a Beckman airfuge. The supernatants were diluted 1:1 with 1% SDS-1% 2-mercaptoethanol-62.5 mM Tris pH 6.8 and applied to 14% polyacrylamide gels (27). Autoradiography was carried out with Kodak X Omat-RP film.

Agarose Gel Electrophoresis

The size of the poly(A⁺)-RNA was determined by electrophoresis on 2% agarose slab gels after denaturing the poly(A⁺)-RNA samples with 1 M glyoxal and 50% DMSO in 10 mM sodium phosphate buffer pH 7.0 as described (12). Electrophoresis was carried out in 10 mM sodium phosphate buffer, pH 7.0 at 30 mA constant current for 5-6 hours. Gels were stained with acridin orange (30 $\mu\text{g}/\text{ml}$).

RESULTS

Characterization of poly(A⁺)-RNA from rat seminal vesicle

When total poly(A⁺)-RNA [poly(A⁺)-RNA_{tot}] was analyzed on a 5-25% linear sucrose gradient in 70% formamide (fig 1), one major peak at 11S was resolved which accounted for approximately 35% of the poly(A⁺)-RNA applied on the

Fig. 1 Linear 5-25% sucrose gradient in 70% formamide of rat seminal vesicle poly(A⁺)-RNA_{tot}.

gradient. Preparative linear 5-20% sucrose gradient run in 1% SDS (fig 2), resulted in a similar major peak [poly(A⁺)-RNA_{11S}] in the 10-12S region. Ribosomal 28S, 18S, and 5S RNA were used as markers. The poly(A⁺)-RNA_{11S} peak was pooled and precipitated. The number average size of poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} was determined by hybridization of ³H-poly(dT) to fractions from linear 5-25% sucrose gradients in 70% formamide (10). The results show that the number average molecular size is 1050 NT and 800 NT for poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} respectively.

Poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} were denatured with DMSO and glyoxal as described in Materials and Methods and analyzed on 2% native agarose gels using ribosomal RNA and chicken β-globin mRNA as markers (fig 3). In both total poly(A⁺)-RNA and poly(A⁺)_{11S} two major bands can be seen, and for poly(A⁺)-RNA_{11S} these two bands represent ~80% of the material applied to the gel. The size of these major bands was estimated to be 620 and 540 NT, respectively by comparison to chicken β-globin mRNA (25) and ribosomal 28 and 18S RNA.

Characterization of ³H-complementary DNA

The synthesis by AMV reverse transcriptase of ³H-complementary DNA (³H-cDNA) to seminal vesicle total poly(A⁺)-RNA (³H-cDNA_{tot}) and to poly(A⁺)-RNA_{11S} (³H-cDNA_{11S}) was at least 96% dependent on the presence of oligo(dT)₁₂₋₁₈ as primer. The ³H-cDNA's were sized on alkaline 5-20% linear sucrose gradients and fractions corresponding to 300-1000 NT were pooled and used for the hybridization studies (fig 4). Both ³H-cDNA's had an average size of 650 NT. Sheared DNA and φX-174 DNA were used as markers.

Homologous hybridizations

The homologous hybridization for ovalbumin mRNA (mRNA_{ov}) (1850 NT) was

Fig. 2 1% SDS, linear 5-20% sucrose gradient of rat seminal vesicle total poly(A⁺)-RNA.

Fig 3. Analysis of RNA denatured with 1 M glyoxal and 50% DMSO and run a 2.5% native agarose gel in 10 mM sodium phosphate buffer pH 7.0. Lane (A) total seminal viscile poly(A⁺)-RNA; (B) poly(A⁺)-RNA_{11S}; (C) ribosomal 28 and 18S RNA; (D) chicken β -globin messenger RNA.

used as a kinetic standard (fig 5). RNA extracted from hen oviducts and containing 0.67% mRNA_{ov} (11) was hybridized to cDNA_{ov} and $C_{rot} \frac{1}{2}$ was found to be $4.5 \cdot 10^{-1}$. After correction for the mRNA_{ov} concentration the $C_{rot} \frac{1}{2}$ for an 100% pure mRNA_{ov} homologous hybridization would be $3.0 \cdot 10^{-3}$. ($0.0067 \times 4.5 \cdot 10^{-1}$).

The hybridization of poly(A⁺)-RNA_{tot} to its ³H-cDNA is shown in fig 5. Approximately 40% of the sequences in the ³H-cDNA probe constitute a highly

Fig. 4 Sedimentation profile on an alkaline 5-20% sucrose gradient of (—) $^3\text{H-cDNA}_{\text{tot}}$ and (---) $^3\text{H-cDNA}_{11\text{S}}$. The gradient was run at $+5^\circ\text{C}$ for 18 h at 38 K in a SW 41 rotor. The bars show the fractions that were pooled and used for the hybridization experiments. Sheared DNA and $\phi\text{X 174}$ DNA were used as markers.

abundant class of sequences with $\text{Crot } \frac{1}{2} = 5 \cdot 10^{-3}$. A $\text{Crot } \frac{1}{2}$ of $5 \cdot 10^{-3}$ corresponds to 2-3 sequences of an average size of 1000 NT (Table 1). Two more classes of sequences were present in $\text{poly(A}^+\text{)-RNA}_{\text{tot}}$. The middle abundant class representing 42% and the low abundant class represent 17% of the sequences in $^3\text{H-cDNA}_{\text{tot}}$. The complexity of these two classes of sequences correspond to

Fig. 5 (▲▲▲) Hen oviduct RNA containing 0.67% mRNA_{ov} hybridized to $^3\text{H cDNA}_{\text{ov}}$. (●●●) $\text{Poly(A}^+\text{)-RNA}_{\text{tot}}$ from rat seminal vesicle hybridized to its $^3\text{H-cDNA}$. Background (5%) was subtracted before calculating the amount of material resistant to S_1 -nuclease.

TABLE I

SEQUENCE COMPLEXITY OF POLY(A⁺)-RNA FROM RAT SEMINAL VESICLE

Total poly(A ⁺)-RNA	frac. of total	Crot $\frac{1}{2}$ obs.	Crot $\frac{1}{2}$ isol. ^a	sequence complexity ^b NT 10^{-5}	# different sequences ^c	copies/cell ^d
	0.41	0.014	0.005	0.031	3	52,000
	0.42	0.23	0.09	1.7	150	1,100
	0.17	4	0.68	4.2	400	160
Poly(A ⁺)-RNA _{11S}	0.85	0.009	0.007	4316	5	30,000

^aCrot $\frac{1}{2}$ obs. x fraction of total^b $\frac{\text{Crot } \frac{1}{2} \text{ isol.}}{\text{Crot } \frac{1}{2} \text{ stand.}} \times 1850 \text{ (mRNA}_{\text{ov}} = 1850 \text{ NT)}$ ^cNumber average size for poly(A⁺)-RNA_{tot} = 1000 NT; for poly(A⁺)-RNA_{11S} = 800 NT^dAssuming 2.2×10^{-7} μg poly(A⁺)-RNA/cell (16) copies/cell = $\frac{\text{RNA} \times \text{frac. of total} \times 6.0 \times 10^{23}}{\text{av. size RNA } 330 = \text{number of sequences}}$

150 (middle abundance) and 400 (low abundance) different sequences of an average length of 1000 NT. It is difficult to accurately estimate the complexity of the high complexity or low abundance class due to the large concentration of the highly abundant class.

When ³H-cDNA_{11S} was back hybridized to poly(A⁺)-RNA_{11S} (fig 6) the highly abundant class of sequences represented 85% of the sequences in the ³H-cDNA_{11S}. The Crot $\frac{1}{2}$ for this highly abundant class is 7×10^{-3} which corresponds to relative few sequences of an average length of 800 NT (Table I). Thus, a vast majority of the poly(A⁺)-RNA_{11S} population (80-85%) represents the highly abundant class of poly(A⁺)-RNA present in poly(A⁺)-RNA_{tot}. The data for the homologous hybridizations are summarized in Table I.

Heterologous hybridizations

When ³H-cDNA to total poly(A⁺)-RNA (³H-cDNA_{tot-SV}) from rat seminal vesicle was hybridized to total rat ventral prostate poly(A⁺)-RNA little or no sequence homology could be observed (fig 6). However a Crot value of 1, 20% of the sequences in ³H-cDNA_{tot-SV} can be saturated by total prostate poly(A⁺)-RNA and at this same Crot, the seminal vesicle total poly(A⁺)-RNA homologous hybridization is complete. This sequence homology among the scarce sequences might represent sequences coding for "housekeeping" functions shared by all cells (12,13).

In vitro translation of poly(A⁺)-RNA_{tot}.

Aliquots from the fractions constituting the poly(A⁺)-RNA_{11S} peak on the 5-20% sucrose gradient were tested in the in vitro translation system and the products were analyzed on polyacrylamide gels and compared to the in vitro translation products of poly(A⁺)-RNA_{11S}. Total polypeptides secreted by the

Fig. 6 (o-o-o) ^3H -cDNA_{11S} hybridized to poly(A⁺)-RNA_{11S} from rat seminal vesicle (Δ-Δ-Δ) ^3H -cDNA_{tot-sv} hybridized to rat ventral prostate total poly(A⁺)-RNA. (●-●-●) Homologous hybridization of poly(A⁺)-RNA_{tot} included as a reference. Background subtracted as mentioned in legend to fig. 5.

seminal vesicle and protein IV (15) isolated from seminal vesicle secretion according to Ostrowski *et al.* (15) were run on the same gel. As can be seen (fig 7) the *in vitro* translation products migrated slightly more slowly than their presumptive corresponding *in vivo* protein. The same difference between *in vitro* synthesized products from mRNA and *in vivo* proteins from rat seminal vesicle has been reported by others (15). The *in vivo* pattern show six dominant protein bands, all of which have been described by Ostrowski *et al.* Based on a molecular weight comparison, the two major protein bands among the poly(A⁺)-RNA_{11S} *in vitro* translation products should correspond to proteins IV and V described by Ostrowski *et al.* (15). The molecular weight of these two bands were estimated to be 17,000 and 14,000 daltons respectively. Fig. 7 also shows that the 620 NT long mRNA enriched for on the heavy side of the poly(A⁺)-RNA_{11S} peak synthesizes predominantly the 1700 dalton protein.

DISCUSSION

The highly abundant class of poly(A⁺)-RNA from rat seminal vesicle [poly(A⁺)-RNA_{11S}] was isolated by preparative sucrose gradients in SDS. Poly(A⁺)-_{11S} and poly(A⁺)-RNA_{tot} were analyzed on 2% native agarose gels under denaturing conditions gels and two major bands were detected. By using c-DNA techniques the sequence information in poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} was investigated.

Fig 7. Polyacrylamide gel analysis of in vivo proteins and in vitro translation products from rat seminal vesicle. Lane (A) abundant proteins in rat seminal vesicle secretion numbered I-V according to (15); (B) protein IV purified according to (15); autoradiograms of in vitro translation products of (C) poly(A⁺)-RNA_{11S}; and (D) the 620 NT long poly(A⁺)-RNA enriched for on the heavy side of the poly(A⁺)-RNA_{11S} peak.

The results revealed the presence of a highly abundant class of sequences corresponding to 3-5 species of poly(A⁺)-RNA and representing 41% and 85% of the poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} respectively. These results were compared to the results from in vitro translation of poly(A⁺)-RNA_{11S} and the 620 NT long poly(A⁺)-RNA enriched on the heavy side of the poly(A⁺)-RNA_{11S} peak.

The poly(A⁺)-RNA isolated by a repeated oligo(dT)-cellulose chromatography (for details see methods), as judged by the agarose gel analysis (fig 3) contained very little ribosomal RNA.

The number average size was found to be 1000 NT and 800 NT for poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S}, respectively. Higgins *et al.* (1979) have reported 600 NT as the number average size of seminal vesicle poly(A⁺)-RNA_{tot}. The number average size for poly(A⁺)-RNA from most tissues studied (17, 18, 19, 20, 10) is 1300-1800 NT. The small number average size of seminal vesicle poly(A⁺)-RNA_{tot} (600-1000 NT) reflects the relatively small size of a few

poly(A⁺)-RNA's constituting almost 50% of the total poly(A⁺)-RNA population.

The validity of c-DNA techniques is based on the assumption that all classes of sequences in the template are represented in the c-DNA. Hastie and Bishop (21) have presented evidence that such an assumption is probably correct. The hybridization data revealed that 41% of the sequences in ³H-cDNA_{tot} represented a highly abundant class of poly(A⁺)-RNA representing two to three different sequences present in 52,000 copies/cell. A similar result has been reported by Higgins *et al.* (16). The back-hybridization of poly(A⁺)-RNA_{11S} to its ³H-cDNA indicate one highly abundant class representing 85% of the poly(A⁺)-RNA_{11S} population. The calculation of number of sequences and copies/cell reveals that some sequences, presumably from the middle abundant class, contaminates the highly abundant class of sequences in poly(A⁺)-RNA_{11S}, since the number of sequences increased from 3 to 5 and copies/cell decreased from 50,000 to 30,000 when compared to the highly abundant class in poly(A⁺)-RNA_{tot}.

It has been shown that poly(A⁺)-RNA from rat ventral prostate contains a large abundant class of sequences (5, 6). Parker *et al.* (6) has shown that the isolated abundant class ³H-cDNA made from total prostate poly(A⁺)-RNA does not react with poly(A⁺)-RNA from seminal vesicle. The hybridization of ³H-cDNA_{tot-SV} to total rat ventral prostate poly(A⁺)-RNA (fig 6) also indicates little or no sequence homology among the highly abundant sequences. From these data mentioned above it is clear that the highly abundant class poly(A⁺)-RNA present in the sex-accessory organs is tissue specific.

The hybridization data of poly(A⁺)-RNA_{tot} and poly(A⁺)-RNA_{11S} to their respectively c-DNA's were supported by analysis of the RNA's on 2% native agarose gels (fig 3). Poly(A⁺)-RNA_{tot} revealed 2-3 major bands and the same 2-3 bands were also the main components in poly(A⁺)-RNA_{11S} (fig 3). These bands represented 70-80% of the material applied to the gel. Thus the agarose gel analysis together with the hybridization data indicate that poly(A⁺)-RNA_{11S} represent a partial purification of the highly abundant class of poly(A⁺)-RNA present in poly(A⁺)-RNA_{tot}.

When poly(A⁺)-RNA_{11S} was translated in a cell free wheat germ extract two major protein bands were detected with molecular weights in the 14,000-17,000 dalton region (fig 7). Similar results were reported by Ostrowski *et al.* when they translated seminal vesicle total poly(A⁺)-RNA (15). Since the major proteins most likely are coded for by the mRNA's belonging to the abundant class this would indicate that the vast majority of poly(A⁺)-RNA_{11S} contain information corresponding to these two polypeptides. Furthermore,

the in vitro translation of the heavy side of the poly(A⁺)-RNA_{11S} peak shows that the 620 NT mRNA codes for the 17,000 dalton protein. This result also suggests that the 540 NT mRNA codes for the 14,000 dalton protein. This conclusion is again supported by the hybridization data and the agarose gel analysis. Fig 7 also shows that the corresponding protein bands isolated from seminal vesical secretion have a slightly lower molecular weight. As have been reported (15) this may indicate that the proteins in the seminal vesicle like many other secretory proteins are synthesized in a precursor form containing a NH₂-terminal extension.

By comparing the in vitro and in vivo protein patterns (fig 7) and previously discussed results (15) we conclude that the 17,000 dalton protein coded for by the 620 NT mRNA is a precursor to protein IV. These results also indicate that the protein V precursor (14,000 dalton) is coded for by the 540 NT mRNA. Ostrowski *et al.* (15) also showed that antibodies prepared to protein IV precipitated 20% of the acid-precipitable material from the in vitro translation of poly(A⁺)-RNA_{tot} and that testosterone induced a significant increase in the synthesis of proteins IV and V. Protein IV and V have also been described by others (8).

The data presented here with respect to poly(A⁺)-RNA_{11S} together with the testosterone dependance of proteins IV and V as mentioned above indicate that the highly abundant class of poly(A⁺)-RNA from rat seminal vesicle may offer a useful system for studying the effect of testosterone on gene regulation.

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