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TWO LOW MOLECULAR WEIGHT NUCLEAR RNAS, ISOLATED FROM AVIAN ERYTHROBLAST NUCLEAR RIBONUCLEOPROTEIN COMPLEXES, HYBRIDIZE TO DUCK PRE-MESSENGER AND GLOBIN MESSENGER RNA.

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SUMMARY: Low molecular weight RNAs (lmw RNA) extracted from isolated small nuclear ribonucleoprotein complexes (snRNP) of purified duck erythroblast nuclei were enzymatically labeled in vitro at the 5' terminus using (^{32}P)-ATP and T_4 polynucleotide kinase (1). Hybridization of these labeled lmw RNA species to purified fractions of duck nuclear and cytoplasmic RNA revealed 2 principal RNA species of approximately 27,000 and 58,000 molecular weight which hydrogen-bonded to nuclear pre-messenger RNA (both poly(A)$^+$ and poly(A)$^-$) and to purified cytoplasmic globin messenger RNA.

INTRODUCTION

Investigations over the past ten years have revealed a unique class of discrete low molecular weight nuclear RNAs designated small nuclear RNA or snRNA (2-6). Experiments have demonstrated that these RNA sequences are found in the eukaryotic nucleus as RNA constituents of various nuclear RNA:protein structures including small nuclear ribonucleoprotein particles (snRNP)(7-10), pre-messenger ribonucleoprotein particles (pre-mRNP)(11-13), and the residual nuclear matrix or nuclear skeleton (14-16). The association of snRNP, and in particular snRNA, with pre-messenger RNA sequences (10) has suggested a possible role for these lmw RNAs in the processing and/or transport of nuclear RNA.

Recent models for the processing of viral (17,18) as well as eukaryotic messenger RNA (19) have proposed the involvement of lmw RNAs in the splicing of precursor molecules through intermolecular base pairing of lmw RNA sequences

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to the region around the splice joints of the pre-messenger molecule. Indeed, the finding that in CHO cells a 4.5 S RNA is found hydrogen-bonded to pre-mRNA and mRNA (20) supports this concept of base pairing between lmw RNA sequences and messenger RNA. The association of polypeptides with snRNA in forming snRNP particles indicates that these complexes might ultimately be important in the establishment of processing complexes necessary for RNA maturation. In the work reported here, we have examined the ability of isolated lmw nuclear RNAs to hydrogen-bond to various populations of duck erythroblast nuclear and cytoplasmic RNA.

MATERIALS AND METHODS

Materials. Adenosine (γ-^{32}P) triphosphate (2000 Ci/mmole) was purchased from Amersham England. T_4 polynucleotide kinase and bacterial alkaline phosphatase were obtained from Worthington and tabacco acid pyrophosphatase from Bethesda Research Laboratories.

Preparation and labeling of lmw RNA. Avian erythroblast nuclei were prepared from selected young cells of Peking Ducks (21) and subsequently extracted in in pH 8.0 buffer. Isolated nuclear ribonucleoprotein particles were fractionated on sucrose gradients into pre-messenger RNP (40 S) and small nuclear RNP (10-20 S) fractions (10). Pooled RNP fractions were phenol extracted (22) and the lmw RNA sequences of snRNP or pre-mRNP labeled *in vitro* with (^{32}P)-ATP using T_4 polynucleotide kinase (1). RNA analysis was carried out on 10-15% gradient polyacrylamide slab gels as previously described (10) and these gels were subsequently stained with EtBr or dried and autoradiographed.

Preparation of duck nuclear and cytoplasmic RNAs. Large nuclear RNA (> 15 S) was prepared by fractionation of total nuclear RNA on a Sepharose 2-CL column in 90% formamide as previously described (23) and subsequent chromatography on oligo-dT cellulose into poly(A)$^+$ and poly(A)$^-$ RNA fractions. Globin poly(A)$^+$ 9 S messenger was prepared by chromatography on oligo-dT cellulose of the RNA phenol extracted from isolated 15 S duck globin mRNP particles (24). Cytoplasmic RNA of approximately 18 S in size was isolated from a sucrose gradient (25) after preparation of total RNA by phenol extraction of the 20-45 S region of a sucrose gradient fractionating EDTA-dissociated polyribosomes (24). Total RNA of free or non-polyribosomal associated cytoplasmic mRNP particles was prepared by phenol extraction of the isolated 15-45 S mRNP complexes (24). Duck cytoplasmic small RNA, fractionated on DEAE-cellulose (26), was prepared by phenol extraction of the cytoplasmic supernatant remaining after pelleting free mRNP particles by centrifugation (365,000 x g for 5 hours)(24).

Hybridization of lmw RNAs to duck nuclear and cytoplasmic RNAs. Selected populations of duck erythroblast RNA were covalently linked to Whatmann 540 paper as previously described (27). After pre-incubation of filters (approximately 1 cm^2 in the bottom of a siliconized glass vial) for 6 hours at 37°C in hybridization buffer containing 1% glycine and 1 mg/ml yeast tRNA, approximately 1 x 10^6 cpms of labeled lmw RNAs from snRNP suspended in 50% formamide, 3xSSC, containing 0.02% ficoll and 0.02% polyvinylpyrrolidone were layered on each filter. Filters were incubated for 40-60 hours at 37°C, subsequently washed with 1 ml aliquots of 50% formamide, 3xSSC buffer containing 5 mM EDTA and 0.2% SDS, and then hybridized RNAs eluted at 65°C in 1/2 ml volumes of 90% formamide containing 50 mM Tris-HCl pH 7.4, 5 mM EDTA, and 0.2% SDS. A 1/50 volume of each fraction was counted to determine the radioactivity profiles, and appropriate pools were ethanol precipitated and analyzed on polyacrylamide gels (10).

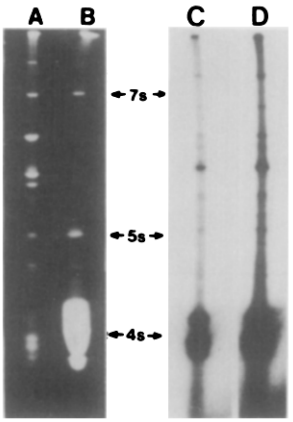

Figure 1. Polyacrylamide gel analysis of in vitro labeled lmw RNA species. (A) Unlabeled lmw RNAs of snRNP revealed by EtBr staining. (B)RNA standards of 25,000 (4 S), 38,000 (5 S), and 110,000 (7 S) molecular weight revealed by EtBr staining. (C)Autoradioagraphy of in vitro (^{32}P)-labeled lmw RNAs of snRNP. (D)Longer exposure of (^{32}P)-labeled lmw RNAs seen in lane C.

RESULTS

In vitro labeling of lmw RNA. Sucrose gradient fractionation of an avian erythroblast nuclear extract yields a 40 S pre-mRNP particle containing primarily pre-messenger RNA (21) and a class of small nuclear RNP complexes composed primarily of lmw RNA species, 4 to 7 S in size (10). Shown in Figure 1A are the lmw RNAs isolated from bulk snRNP particles, a population which includes the previously characterized 17 S and 12 S snRNP complexes (10). Comparison with in vitro labeled lmw RNAs of snRNP (lane C) reveals that a significant number of these lmw RNA species are labeled using this technique. However, the absence and/or disproportionate labeling of some species indicated that either their secondary structure or their phosphatase-resistant 5' "cap" structure effected their ability to be labeled (see Discussion).

Labeled lmw RNA hybridization to nuclear and cytoplasmic RNA. Approximately equal amounts of (^{32}P)-labeled RNA were suspended in hybridization buffer containing 50% formamide and 3xSSC, and subsequently incubated at 37°C with various fractions of duck nuclear and cytoplasmic RNA that had been covalently bound to Whatmann paper as previously described (27). After hybridization, filters were extensively washed in buffer containing 50% formamide and 3xSSC, and then bound RNA eluted in 90% formamide at 65°C. The elution profile of each filter, shown in Figure 2, reveals that while no labeled RNA bound to either the blank or poly(A) filter, each of the remaining 6 filters hydrogen-bonded varying amounts of labeled lmw RNA. In each hybridization series, the nuclear RNA populations consistently bound 3 fold more (^{32}P)-labeled lmw RNA than any of the cytoplasmic RNA fractions.

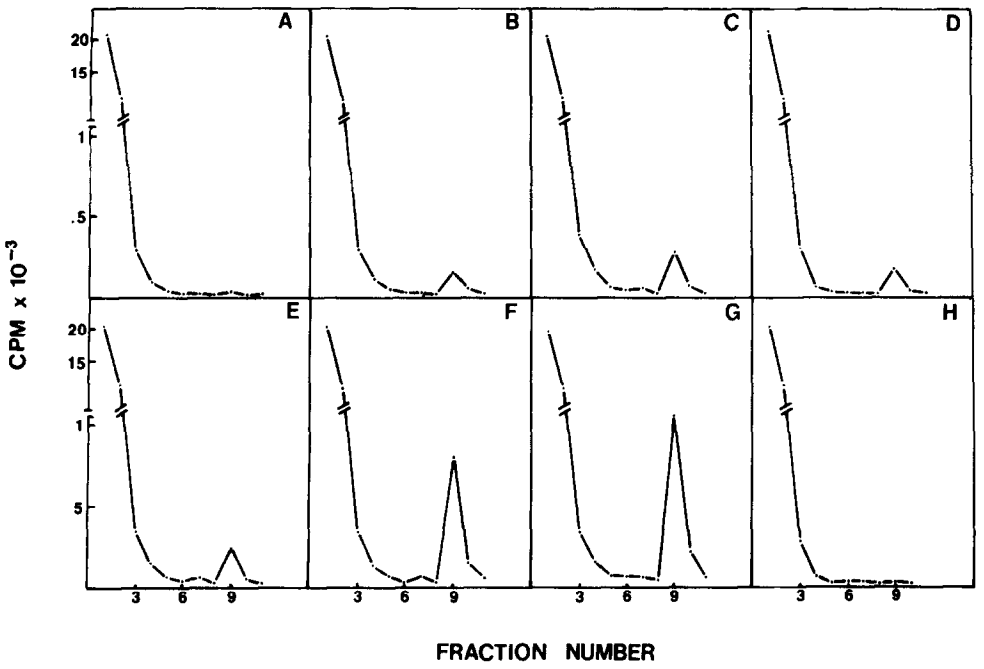

Figure 2. Hybridization of (^{32}P)-labeled lmw RNAs to various populations of duck erythroblast nuclear and cytoplasmic RNA. Each filter, containing 10-15 ug of covalently attached RNA, was incubated with labeled lmw RNA. Elution profiles seen in Figure 2 represent the released radioactivity when filters were washed with buffer containing 50% formamide and 3xSSC (fractions 1-8), and then with 90% formamide (fractions 9-11). Covalently linked RNA populations were; (A)Blank filter or no RNA. (B)Small duck cytoplasmic RNA. (C)Polyribosomal 18 S RNA. (D)Total RNA of free or non-polyribosomal associated cytoplasmic mRNP particles. (E)Globin 9 S messenger RNA (poly(A)$^+$). (F)Large, poly(A)$^+$ nuclear RNA (>15 S). (G)Large, poly(A)$^-$ nuclear RNA (> 15 S). (H)Commercially obtained poly(A) sequences.

Analysis of the hybridized lmw RNA species from these filters on a polyacrylamide slab gel (Figure 3) revealed 2 principal lmw RNAs of approximately 27,000 and 58,000 molecular weight. The absence of hybridized RNAs on the blank or poly(A) filters as well as the lack of hybridized 4 S RNA (the most heavily labeled small RNA of this total population as seen in Figure 1C) was indicative of the true nature of the hybrids formed.

The hybridization of these 2 RNAs to nuclear RNA (specifically poly(A)$^+$) and to cytoplasmic globin 9 S messenger RNA clearly demonstrated their sequence complementarity to message-containing nuclear and cytoplasmic RNA. The ubiquitous hybridization of these same species to several cytoplasmic RNA filters, most notably filters containing small cytoplasmic RNA and polyribosomal 18 S RNA, made interpretation of results in these cases more complex. However, examination of the protocols used to prepare these cytoplasmic RNAs reveals that stringent conditions (i.e. oligo-dT cellulose chromatography) to remove mRNA

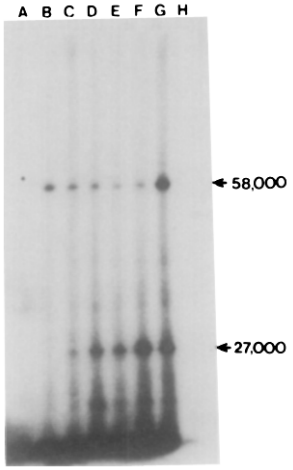

Figure 3. Low molecular weight RNA species hybridized to duck nuclear and cytoplasmic RNA populations. The lanes labeled A-H in this Figure represent the RNAs eluted in 90% formamide from the corresponding filters designated A-H in Figure 2 whose covalently attached RNA populations were; (A)Blank filter. (B)Small cytoplasmic RNA. (C)Polyribosomal 18 S RNA. (D)RNA of free mRNP particles. (E)Globin 9 S mRNA. (F)Large nuclear RNA, poly(A)$^+$. (G)Large nuclear RNA, poly(A)$^-$. (H)Commercially obtained poly(A) sequences. Hybridized RNAs were analyzed on a polyacrylamide slab gel and the dried gel autoradiographed.

sequences were not utilized. It is therefore likely that each cytoplasmic RNA population contains mRNA sequences. The fact that 9 S globin mRNA or its possible breakdown products would not be present on filter C suggests that the hybridization of these two lmw RNA species is not specific for only globin mRNA sequences. It is unclear at the present time why only the 58,000 molecular weight RNA hybridizes to small cytoplasmic RNA.

The previous observation that the CHO cell 4.5 S RNA which was capable of hybridizing to messenger RNA could not be found in isolated polyribosomes (20), prompted us to investigate the capability of these hybridizing RNAs to hydrogen-bond with messenger RNAs purified from free or non-polyribosomal associated mRNP as well as polyribosomal mRNP (filters D and E). The hybridization of both the 27,000 and 58,000 molecular weight RNAs to both populations of mRNP-contained RNA indicates that the *in situ* environment, perhaps the differing RNP nature of each particle class, is responsible for the cellular partitioning of these hybridizing lmw RNA species.

Differential distribution of lmw RNAs in RNP. Previous experiments characterizing duck erythroblast pre-mRNP have shown that lmw RNAs comprise 10-20% of this particle's total RNA moiety (10). To evaluate the nuclear distribution of these 2 hybridizing lmw RNAs among nuclear RNP, total 40 S pre-mRNP RNA was labeled at the 5' terminus with (^{32}P)-ATP and subsequently hybridized to various

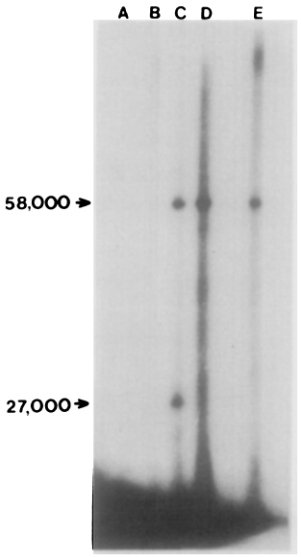

Figure4. Differential distribution of hybridizing lmw RNAs in duck erythroblast nuclear RNP complexes. Hybridized RNAs were analyzed on a polyacrylamide slab gel and the dried gel autoradioraphed. (A)Labeled lmw RNA of snRNP hybridized to E. coli RNA. (B)Labeled lmw RNA of pre-mRNP hybridized to E. coli RNA. (C)Labeled lmw RNA of snRNP hybridized to duck nuclear RNA (poly(A)$^-$). (D)Labeled lmw RNA of pre-mRNP hybridized to duck nuclear RNA (poly(A)$^-$). (E)Labeled lmw RNA of pre-mRNP hybridized to duck globin 9 S messenger RNA (poly(A)$^+$).

RNA filters. Analysis of hybridized RNAs on a polyacrylamide gel (Figure 4) revealed that while no lmw RNA species from either particle hybridized to control filters containing E. coli RNA, both particle classes contained the larger 58,000 molecular weight RNA species which hybridized to filters containing nuclear RNA. In contrast, the smaller 27,000 molecular weight RNA was detected only in the lmw RNAs isolated from snRNP. Due to the back-hybridization of labeled pre-mRNA sequences to filter D containing covalently linked nuclear RNA, this result is more clearly seen in lane E with the hybridization of labeled lmw RNAs from pre-mRNP to purified globin mRNA.

DISCUSSION

These experiments show that 2 lmw RNA species of 27,000 and 58,000 molecular weight hybridize to message-containing RNA sequences. Due to the difficulty in labeling some lmw RNA sequences at the 5' terminus, it is possible that the 2 species described in these experiments are only a subset of possible hybridizing lmw RNAs. In fact, identical experiments in mouse ascites cells using lmw RNAs labeled at the 3' end indicate that this is indeed the case (Maxwell and Martin, unpublished results).

At present, the precise identity of these duck lmw RNAs has yet to be established. The smaller 27,000 molecular weight RNA is similar in size and hybridization characteristics to the CHO cell 4.5 S lmw RNA reported by Jelinek and Leinwand (20), while the larger RNA species of 58,000 molecular weight has the same electrophoretic mobility as duck erythroblast lmw RNA species U_{1a}. Similar experiments performed in mouse ascites cells (Maxwell and Martin, unpublished results) have also shown that an RNA species which co-migrates with U_{1a} can be labeled *in vitro* and subsequently can hybridize to nuclear pre-mRNA and cytoplasmic mRNA in a manner entirely analogous to that described here.

In view of the recent speculation concerning the role of U_{1a} as a guide RNA in the elimination of intervening sequences during pre-mRNA processing (19), it is of particular importance to have shown that the 58,000 molecular weight species can also hybridize to a specific mature mRNA. The implication from this observation is that at least some sequence(s) outside the intron regions can also become involved in hydrogen-bonding with lmw RNA. In this respect, the hybridization of the 58,000 molecular weight species resembles that recently reported to occur between VA_{II} RNA of adenovirus infected cells and viral fiber mRNA (28). While the proposed model for U_{1a} involvement in RNA splicing is indeed attractive, the site of hybridization has still to be determined experimentally. Further resolution of this question awaits detailed mapping experiments.

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