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Non-random localization of ribonucleoprotein (RNP) structures within an adenovirus mRNA precursor

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ABSTRACT

Heterogeneous nuclear protein complexes (hnRNP) containing the precursor RNA from the adenovirus early region 2 were analysed to determine the specificity of protein-RNA interaction. RNA precursor sequences were present in isolated hnRNP complexes and endogenous 30S particles. At least 20 - 40 bases long fragments were protected when RNase A was used to remove unprotected RNA sequences in hnRNA complexes. Similarly around 40 bases of RNA were protected in 30S particles. These sequences represent discrete regions of the adenovirus genome. Especially sequences complementary to the EcoRI-F fragment encoding the first leader and the major intron for the DNA binding protein (DBP) RNA precursor, were analysed in detail. Tentatively, sequences resistant to RNase A were located in the middle of the intron and at the splice-donor junction of the first leader of the DBP precursor RNA. The same sequences were identified irrespective whether hnRNP complexes or 30S particles were used suggesting that 30S particles originate from hnRNP complexes. A 38.000 dalton protein appears to be in direct contact with RNA sequences complementary to the EcoRI-F fragment.

INTRODUCTION

The nascent rapidly labelled heterogeneous nuclear RNA (hnRNA) of eukaryotic cells is associated with nuclear proteins in structures called heterogeneous nuclear ribunocleoprotein (hnRNP) complexes (1). In the electron microscope these complexes probably correspond to beaded, relatively uniform particles spaced along the RNA chain (2). Similar particles, measuring 20 - 30 nm in diameter, can be prepared from isolated nuclei after digestion with endogenous RNases or proteases followed by analysis on sucrose gradients (3,4,5). These particles sediment between 30 and 40S and contain a heterogeneous set of polypeptides dominated by proteins around 31,000 - 42,000 daltons (4,5). Because of the low complexity of the dominant polypeptides and the conserved nature of the protein pattern in between species, no specific RNA sequences have been postulated to be confined to the particle structure (6 and references cited therein). On the other hand, it was recently reported that the

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particles are non-randomly distributed along the growing RNA chain from a single gene as visualized from chromatin spreads of Drosophila embryos (7). It has, however, not been established whether the particle structures in the electron micrographs correspond to 30S particles purified from sucrose gradients.

A direct molecular approach is now technically feasible in studying the hnRNP structures. The presence of globin mRNA precursor molecules in purified hnRNP complexes has been established (8). More detailed analysis regarding the RNA-protein interaction at specific regions along an mRNA precursor has not yet accumulated. Steitz and Kamen (9) reported that stringent RNase treatment of hnRNP containing polyoma virus mRNA precursor sequences leaves preferentially the coding regions protected as revealed by fingerprint analysis.

A more detailed study of the hnRNP structure may help to understand the mechanism of RNA processing and the structure and function of 30S particles. We have in this study attempted to map where RNA and protein interact within a single mRNA precursor RNA species, by utilizing the adenovirus system, where the DNA sequence, splicing patterns and high abundance of mRNA precursors are available. For these studies we have selected the early adenovirus E2 region which encodes a single mRNA precursor extending from around 75 to 62 map units on the leftward transcription strand of the adenovirus genome (for a review see 10). The mRNA from this region encodes a DNA binding protein (DBP) which is required for viral DNA replication (11).

MATERIALS AND METHODS

Cell culture and adenovirus infection

The maintenance of HeLa spinner cultures and the procedure for adenovirus type 2 infection followed published protocols (12). Infection was performed at a multiplicity of 1,000 fluorescence focus forming units (FFU) per cell. In all experiments cells were harvested at 3 hrs post infection and a 5,6-^{3}H-uridine (New England Nuclear Corp.) pulse (15 min at 10 µCi/ml of spinner culture) was introduced before harvest allowing us to monitor nuclear RNP complexes during preparation.

Purification of nuclear RNP complexes

HeLa cells, washed several times in PBS, were lysed in homogenisation buffer (0.1 M Tris-HCl, pH 7.9, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 6 mM ß-mercaptoethanol, 0.4% NP-40 and proteolytic inhibitors (see below) for 10 min on ice. The nuclei were recovered by centrifugation at 240 g for 5 min,

followed by two washes in sonication buffer (homogenisation buffer without NP-40). In the homogenisation and sonication media we added the following proteolytic inhibitors: leupeptin, antipain, pepstatin (all at 0.1 µg/ml final concentration, Protein Research Foundation, Japan), N-lysine chloromethyl ketone, L-1-tosylamide 2-phenyl ethylchloromethyl ketone (10 µM final concentration, Sigma Chemical Co.) and phenyl methylsulfonyl-fluoride (0.05 mg/ml, Fluka). HnRNP complexes were purified from nuclei broken by sonication for two two-second pulses. Following removal of nucleoli and unbroken nuclei by centrifugation at 5,500 g for 10 minutes on top of a 30% sucrose cushion in a Sorvall swing-out rotor, the nuclear sap was loaded onto stepwise and isokinetic sucrose gradients as indicated in the figure legends. The 30S particles were prepared as previously described (3).

RNase A digestion of hnRNP complexes

Freshly prepared RNP complexes were digested with pancreatic RNase (RNase A) in 40 mM Tris-HCl, pH 7.9, 40 mM NaCl, and 5 mM $MgCl_2$ at 37°C for 20 min at different concentrations of the enzyme. After addition of 10 mM EDTA, 0.5% SDS, and 2 mg/ml proteinase K (final concentrations) the samples were incubated at 0°C for 30 min and then extracted with phenol-chloroform and precipitated by ethanol. The phenol-chloroform step was repeated twice prior to the 5'-end labelling of the RNA. Undegraded hnRNA was digested to yield 5' hydroxyl groups with 2 units of micrococcal nuclease/µg RNA at room temperature for 30 seconds whereafter the reaction was terminated with 10 mM EGTA, yielding adenovirus-encoded nuclear RNA molecules ranging from 20 to 500 bases. Protein-free hnRNA served as a control which under the same conditions was completely degraded with these concentrations of RNase.

5'-end labelling of hnRNA

The RNA samples (0.5-2 µg) were dissolved in polynucleotide kinase buffer (13) and 100 µCi γ-^{32}P-ATP and 1 unit of RNAse-free T_4 polynucleotide kinase (New England Nuclear Corporation) was added. After incubation at 37°C for 1 hour the samples were extracted with phenol-chloroform, chromatographed on a Sephadex G-50 column and ethanol precipitated. The RNA (specific activity: 1-10x10^6 cpm Cẽrẽnkov/µg RNA) was redissolved and used directly for hybridization without a denaturation step.

Blotting and hybridization procedures

Southern blots of EcoRI-cleaved ad2 DNA separated by electrophoresis in 1% agarose gel was carried out according to Southern (14). Transfer of RNA preparations separated on 1.4% methyl-mercury agarose gels were performed according to Alwine et al. (15). AluI-cleaved EcoRI-F fragments were sepa-

rated on a 10% polyacrylamide gel with around 2.5 µg of digested DNA from a clone of the EcoRI-F fragment per lane. The DNA was then transferred to DBM paper by electrophoresis. Using radioactive DNA markers approximately 10-30% of input DNA was covalently linked to the cellulose paper. End-labelled RNA and nick-translated DNA fragments were hybridized to strips of blotted nucleic acids in 50% formamide, 0.9 M NaCl, 5 mM EDTA, 1xDenhardt, 0.5% SDS, 0.2 mg/ml denatured calf thymus DNA, and 0.5 mg/ml yeast RNA at 41°C for 1-2 days. All blots were washed after hybridization in 2xSSC for 8x1 hour at 37°C and dried prior to exposure to Kodak XR-5 films.

Protein analysis

Proteins from the 55-60% sucrose layer were precipitated by acid and analyzed on 10% SDS-polyacrylamide gels. The bands were visualized with the Coomassie Brilliant Blue stain or by fluorography.

Genomic clones

The EcoRI-B and F fragments of ad2 DNA were purified by agarose gel electrophoresis and ligated to the EcoRI-cleaved pBR322 plasmid in P1 facilities. Plasmid DNA were purified from cleaved lysates by either CsCl-gradient centrifugation (16) or aqueous two-phase partition (17).

Hybridisation-selection of crosslinked RNA-protein complexes

Crosslinking of RNA to protein in adenovirus infected cells were carried out 3 hours postinfection using UV-irradiation according to published procedures (18,19). Nuclear polyadenylated RNA with covalent protein was also purified according to these procedures. The RNA-protein complexes were hybridised for 6 days to DBM-paper blots of a EcoRI-restricted Eco-RI-F clone of ad2 DNA in pBR322 separated on a 1% agarose gel. The blot was washed for 2x1 hours with 6xSSC, for 2x1 hours with 2xSSC and for 2x1 hours with 0.1xSSC containing 0.5% SDS at 37°C. After drying the paper, the pBR322 and the EcoRI-F fragments were separately excised and the RNA-protein complexes eluted with 90% formamide, 0.5% SDS and 5 mM EDTA at 70°C for 5 min. Following ethanol precipitation the RNA-protein samples were first treated with 2 µg/ml of RNase A and 2U/ml of T1 RNase and then incubated with ^{32}p-γ-ATP and T4 polynucleotide kinase as above to label the 5'-end of the oligonucleotides remaining linked to the protein. The samples were then extracted with phenol whereupon the buffer phase was replaced several times to remove RNA molecules not associated with proteins. The protein-oligonucleotide complexes accumulated in the phenol phase or at the interface. Finally, the phenol layer and the interface was TCA-precipitated to recover the proteins. The precipitates were dissolved in application buffer and analysed by

SDS-polyacryalmide gel electrophoresis.

RESULTS

Preparation of hnRNP

HnRNP complexes from adenovirus-infected cells were first characterized with regard to protein composition .

After ^{3}H-uridine pulse-labelling of HeLa cells early in ad2 infection (15 minutes at 3 hours), the nuclei were purified and sonicated. A nuclear sap was separated on a discontinous sucrose gradient. HnRNP, containing more than 95 percent of radioactivity incorporated, accumulates at the 55-60% sucrose boundary (Fig. 1A) but when this material was analyzed on an isoki-

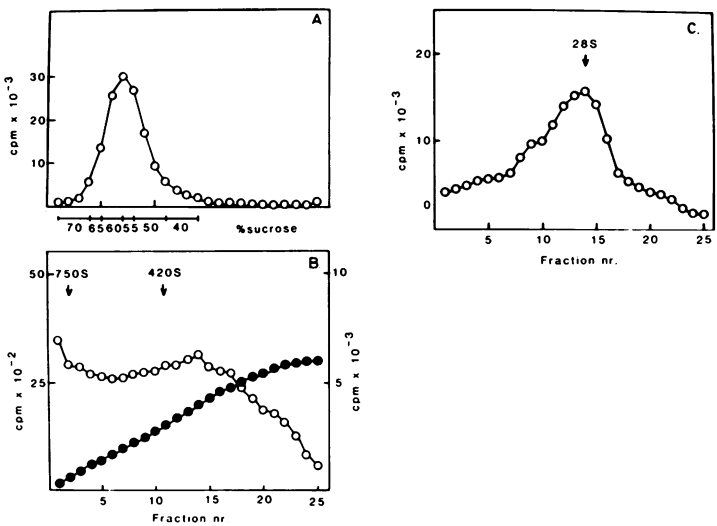

Figure 1. Sucrose gradient analysis of ^{3}H-uridine-labelled nuclear RNP complexes from ad2-infected HeLa cells.
(A) A sonicated nuclear sap from ^{3}H-uridine-labelled (15 min pulse) HeLa cells collected at 3 hours post infection, were applied onto a discontinuous sucrose gradient. Following centrifugation at 18,000 rpm for 12 hours the gradient was fractionated and acid insoluble ^{3}H-uridine monitored (o).
(B). The hnRNP complexes, which sedimented at the 55-60% sucrose layer in (A) were pooled and applied to an isokinetic 21-41% sucrose gradient. After centrifugation at 30,000 rpm for 1.5 hours (including acceleration time), the fractions were analysed as above (o). Filled dots represent accumulated radioactivity by adding cpm from each fraction from left to right thereby obtaining a mean sedimentation value of around 420S.
(C) 30S particles were purified from a nuclear extract (see Materials and Methods) and the sample was applied onto a 15-35% sucrose gradient and spun at 22,000 rpm for 15 hours. 28S rRNA was used as a marker (arrow).

netic sucrose gradient using ad2 as a sedimentation marker (~750S) a more heterogeneous profile was observed with the label sedimenting from 50 to 750S (Fig. 1B).

SDS-PAGE analysis of the peak material in Fig. 1A displays a complex pattern with several polypeptide bands (Fig. 2, lane a). To identify the proteins associated with RNA , hnRNP complexes were treated with low amounts of pancreatic RNase and the material sedimenting at the 55-60% sucrose boundary was reisolated. Analysis of this material (Fig. 2, lane b) revealed that two dominating bands migrating at around 38,000 and 41,000 daltons have disappeared. Proteins in the same size range were also released by the addition of 0.7M NaCl (Fig. 2, lane C). These polypeptides therefore appear to be associated with RNA in ad2-infected HeLa cells early after infection and similar results were also observed for hnRNP from uninfected HeLa cells (not shown). They probably correspond to the C proteins detected by other investigators (19) although with our markers we arrive at slightly smaller molecular weights. Our hnRNP preparations contain only few RNA-associated polypeptides with a molecular weight below 38,000 daltons which may be due to the use of protease inhibitors during preparation of the hnRNP (see Materials and Methods).

Nuclear accumulation of precursor mRNA early in adenovirus infection

Kitchingham and Westphal (20) reported based on heteroduplex analysis in the electron microscope that the majority of the nuclear RNA from the early region E2 was in a precursor mRNA form. This region of the adenovirus genome encodes a 72,000 daltons DNA-binding protein (DBP). The two genomic DNA

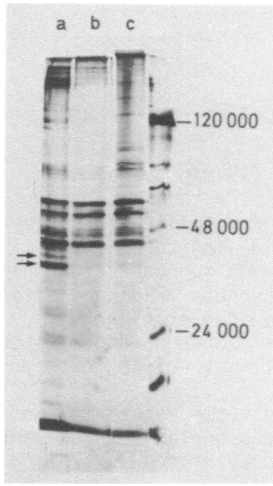

Figure. 2. SDS-PAGE analysis of hnRNP complex and RNA binding proteins. (A) The peak material in Fig. 1A was analysed on a 10% polyacrylamide gel. (B) Protein pattern of hnRNP complexes that had been exposed to RNase A and reisolated as in Fig. 1A. (C) Same as (B), but after treatment with 0.7M NaCl. The protein marker derives from ^{35}S-methionine-labelled ad2 virions. The arrows indicate specific bands lost after RNase and high salt treatments.

fragments (EcoRI-B and EcoRI-F) include respectively exon and intron regions of the DBP-mRNA precursor, thus allowing distinction between spliced and unspliced RNA molecules (see Fig. 7 for a genomic map of the precursor RNA). Accumulation of precursor mRNA in the nucleus was studied by transfer of RNA to DBM filters and probing with radiolabelled cloned DNA fragments (15). Cytoplasmic and nuclear RNA from ad2-infected HeLa cells at 3 hours post infection were purified on oligo(dT) cellulose and analyzed using a radiolabelled EcoRI-B clone as probe. Fig. 3 shows that the majority of the polyadenylated RNA in the nucleus is of the size of the precursor mRNA which has been reported to be 28S (21). Cytoplasmic RNA contained predominantly the fully spliced 18S mRNA for DBP (Fig. 3). The results suggest that around two-thirds of the nuclear polyadenylated RNA from the early E2 region in contrast to other early adenovirus transcription units is in the form of the precursor mRNA at this time of the infectious cycle. The E2 region is therefore well suited for studying the arrangment of hnRNP.

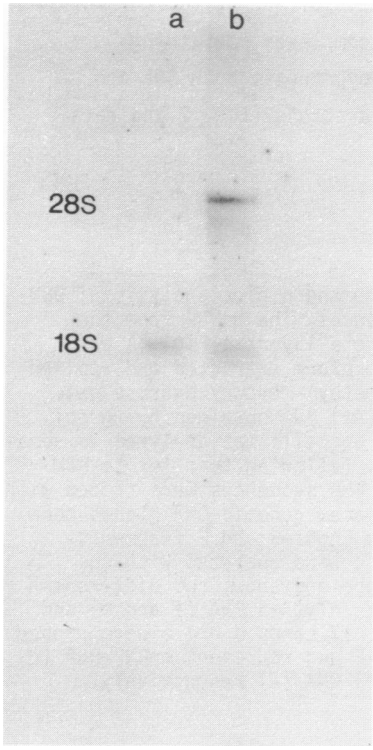

Figure 3. Northern blot analysis of nuclear and cytoplasmic polyadenylated DBP-RNA. The RNA samples were prepared from adenovirus 2-infected HeLa cells at 3 hours after infection as described in Materials and Methods. After separation on 1.4% methyl-mercury hydroxide agarose gels and transfer to DBM-paper the DBP-RNA sequences were visualized by hybridization with internally labeled ^{32}P-EcoRI-B originating from a recombinant clone.
(A) 1 µg of cytoplasmic poly(A+) RNA.
(B) 5 µg of nuclear poly(A+) RNA.

Viral RNA in hnRNP

As demonstrated above the majority of polyadenylated nuclear RNA is in the form of unspliced precursor mRNA of the adenovirus DBP protein. Before analyzing the location of the protein moiety on the mRNA precursor it was also necessary to identify RNA sequences represented in isolated hnRNP. The peak material at the 55-60% sucrose boundary (Fig. 1A) was extracted with phenol-chloroform and analyzed on denaturing gels. After transfer to DBM-paper, sequences present in hnRNP from the E2 region of the adenovirus genome was detected by hybridizing radiolabeled EcoRI-B and EcoRI-F clones to the blots. As a control total cellular poly(A+) RNA from ad2-infected cells was prepared early in infection. Only fully spliced 18S mRNA from early region E2 could be detected in total cellular poly(A+) RNA after a short exposure (Fig. 4, lane a). Only trace amounts of the unspliced 28S molecules could be seen with the Eco-RI-B probe after long exposure (Fig. 4, lane b). When the EcoRI-F fragment was used as probe the 28S precursor mRNA was readily detected in total cellular poly(A+) RNA (Fig. 4, lane e). Fig. 4 (lanes c and d) demonstrates also that the RNA extracted from hnRNP is heterogenous in size varying from 28S to 15S with some accumulation of RNA at the 28S level. These results may suggest that we have captured complexes containing nascent RNA strands (4). In agreement with this hypothesis both DNA and histone proteins were observed in the hnRNP preparations (Fig. 2 and data not shown).

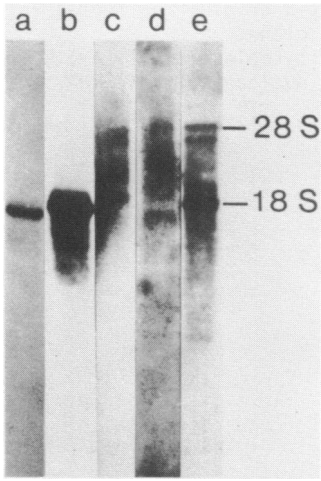

Figure 4. Northern blot analysis of DBP-hnRNA sequences. The hnRNP from the 55-60% sucrose layer in Fig. 1A was phenol-chloroform extracted and applied to a 1.4% methyl-mercury agarose gel. Total poly(A+) RNA obtained by phenol extraction of cells was analyzed in separate slots. Following transfer to DBM-paper, the RNA sequences were probed with nick-translated genomic DNA clones containing the EcoRI-B and F fragments. Lanes a to c were analyzed with the EcoRI-B probe and shows two different exposure of poly(A+) RNA (a and b) and hnRNP-RNA (c). Lanes d and e were probed with EcoRI-F and represent hnRNP-RNA (d) and poly(A+) RNA (e) respectively.

RNase digestion of HnRNP

To establish whether RNA in hnRNP is protected by protein moieties, the complexes were digested with pancreatic ribonuclease (RNase A). Table 1 shows that around 2-3% of the acid-insoluble ^{3}H-uridine label is protected against extensive RNase A digestion in hnRNP complexes and 30S particles under low salt conditions whereas phenol-extracted RNA from these complexes are more sensitive to digestion. These results suggest that RNase resistant regions may be covered by a protective layer of the protein moities in hnRNP.

In order to map the location of the viral RNA regions protected by proteins we initially attempted to use *in vivo* labelled ^{32}P-hnRNA but the amount of label and specific activity was too low for unequivocal assignments. Since RNase A will generate 5-hydroxyl groups, the amount and specific activity of the label in the protected RNA fragment could be enhanced by using T4 polynucleotide kinase and γ-^{32}P-ATP. This approach will however yield a higher specific activity, the smaller the RNA fragment. Therefore stringent RNase A digestion (20 µg/ml) was introduced to avoid contamination of small unprotected oligonucleotides (Table 1). Following this rationale, RNA fragments protected in the HnRNP structure were extracted with phenol and subsequently labeled with polynucleotide kinase Two types of controls were introduced, one consisted of an hnRNA sample subjected to RNase A digestion before end-labelling, the second consisted of hnRNA partially degraded with micrococcal nuclease to obtain random

TABLE 1

RNase resistence of RNA in hnRNP complexes

Sample	Percentage resistence at indicated concentrations of RNase A		
	0.2 µg/ml	2 µg/ml	20 µg/ml
hnRNP complexes	10.7	3.5	2.1
30S particles	N.D.	4.1	2.7
Phenol-chloroform-extracted HnRNA	N.D.	0.7	0.2

hnRNP complexes were isolated from ^{3}H-uridine-labelled (15 min pulse) ad 2-infected cells at 3 hours after infection. Digestion was performed in 100 µl containing 40 mM Tris-HCl pH 7.9, 40 mM NaCl, 5 mM $MgCl_2$ at an RNA concentration of 10 µg/ml for 20 minutes at 37°C with indicated concentrations of RNase A. Samples without RNase A correspond to 100 percent resistance.

N.D. = Not done

fragments of sufficient size for hybridization. The labelled RNA samples were hybridization-selected on ad2 DNA bound to DBM filters as described in Materials and Methods. Analysis on a 10% polyacrylamide-urea gels (Fig. 5) revealed that ad2-encoded nuclear RNA molecules protected against RNase A in their RNP configuration had a size range from 20 to 90 bases with an average around 40 bases. Approximately the same size range was observed for protected RNA in both hnRNP complexes and 30S particles Undigested 30S particle RNA ranged from 30-1,000 bases and micrococcocal nuclease digested hnRNA was of comparable size. In figure 5, lane c is shown that RNA fragments generated from RNase treated hnRNA could not be selected by hybridisation suggesting that proteins protect specific ad2-encoded RNA fragments longer than 20 bases.

Mapping of protected RNA fragments in hnRNP

The localization of the protected RNA fragments on the adenovirus genome was analyzed by hybridization to enzyme-restricted adenovirus DNA. HnRNP was digested with different concentrations of RNase A followed by extraction of the RNA with phenol-chloroform and 5'-labelling of the fragments with polynucleotide kinase. Labelled RNA was first hybridized to ad2 DNA restricted with EcoRI-endonuclease.

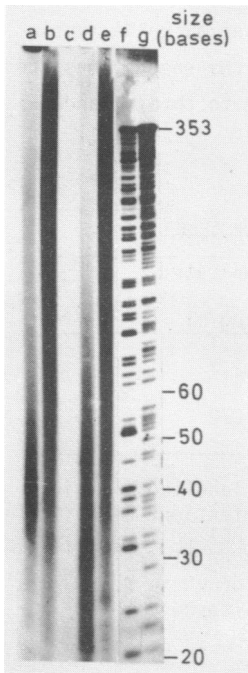

Figure 5. Size analysis of end-labelled adenovirus nuclear RNA
The RNA samples were end-labelled and selected on ad2 DNA, covalently bound to DBM filters (5 µg/filter). The nuclear, adenovirus-specific RNA samples were denatured and analyzed on a 10% urea-polyacrylamide gel.
a) Viral 30S RNP subjected to RNase A (20 µg/ml)
b) Viral RNA fom 30S particles degraded with micrococcal nuclease
c) Protein-free adenovirus hnRNA subjected to RNase A (20 µg/ml)
d) adenovirus hnRNP, subjected to RNase A (20 µg/ml)
e) adenovirus hnRNA degraded with micrococcal nuclease
f and g) Two marker DNA sequence ladders.

Fig. 6 shows that adenovirus hnRNA treated with micrococcal nuclease is complementary to all the DNA fragments including the EcoRI-B and F fragments which contain the sequences complementary to the DBP precursor mRNA. When increasing concentrations of RNase A was used for digestion of the hnRNP, the pattern of hybridization of extracted RNA changed and the hybridization to the EcoRI-A and F fragments are now overrepresented. A similar pattern was observed irrespective whether 30S particles or hnRNP complexes were used for RNase A digestion. There appears to be an overrepresentation of RNA sequences complementary to the EcoRI-D fragment in intact 30S particles which may reflect that the promoter for the E3 region located in the EcoRI-D fragment is very active at early times (20).

It is of interest that the major intron of the DBP precursor mRNA is located in the EcoRI-F fragment (see Figure 7). The position of protein-protected RNA in the EcoRI-F fragment was therefore investigated in more detail. As shown in Fig. 7 the DBP-precursor RNA maps within the EcoRI-B and F fragments on the ad2 genome. The EcoRI-B fragment encodes primarily the main exon while the EcoRI-F fragment encodes the first leader and a major part of the large (2.2 Kb) intron (20) The AluI cleavage map of the EcoRI-F fragment (22) has also been included in Fig. 7. The AluI-I,J and K fragments are located around the first splice junction, where the Alu-J

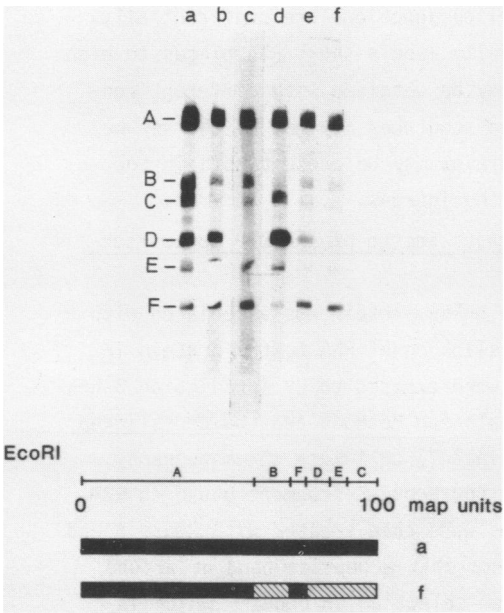

Figure 6. Analysis of RNase A-protected nuclear RNA sequences by hybridisation to EcoRI restricted ad2 DNA. RNA, degraded with micrococcal nuclease are represented in lane a (hnRNA) and lane d (30S particle RNA). Nucleoprotein degraded with 2 µg/ml and 20 µg/ml of RNase A are shown in lanes b and c for hnRNP and in lanes e and f for 30S particles. An EcoRI map of the ad2 genome is shown at the bottom indicating hybridization in lane a and f above.

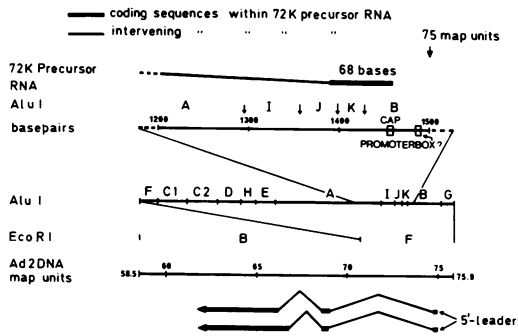

Figure 7. Schematic out-line of the map around the DBP RNA precursor in early region 2 of the adenovirus genome including the AluI map around the first leader 22).

fragment contains 6 base pairs of the leader and 35 of the intron.

In order to identify the position of the protected RNA a blot of a 10% acrylamide gel separating the AluI fragments was made by transferring the fragments electrophoretically to DBM paper. Subsequent hybridization with end-labeled RNA fragments showed that RNA sequences protected against medium amounts of RNase A preferentially hybridize to the Alu-E and H fragments (Fig. 8, lane c). High amounts of RNase A protect in addition RNA sequences hybridizing to the AluI-I and J fragments and to a minor extent to the Alu-A fragment. These results suggest that there exist different forms of RNP structures. At medium concentrations the protected sequences derive mainly from the middle of the intron whereas at high concentrations sequences around the splice junctions are preferentially protected. Since the method preferentially labels short RNA pieces to high specific activity different patterns may be obtained with different concentrations of nucleases. The protected sequences may not of course be present on the same transcript but proteins may be present both in the middle and at the splice junctions of the introns.

HnRNP proteins are associated to the major intron of the DBP precursor mRNA

Finally, in order to establish that an hnRNP protein was associated with the DBP-precursor RNA, UV-induced crosslinking of RNA to the protein in vivo was attempted (23,24) HeLa cells were exposed to UV irradiation 3 hrs after infection as described in Materials and Methods and the crosslinked RNA protein complex was purified by oligo(dT) cellulose chromatography followed by hybridization elution from the EcoRI-F fragment bound to DBM paper. The eluted RNA-protein complexes were then treated with RNase A and subsequently 5'-end labeled. Fig. 9 shows that a peptide band at around 38,000 daltons could be identified after selection on EcoRI-F which is

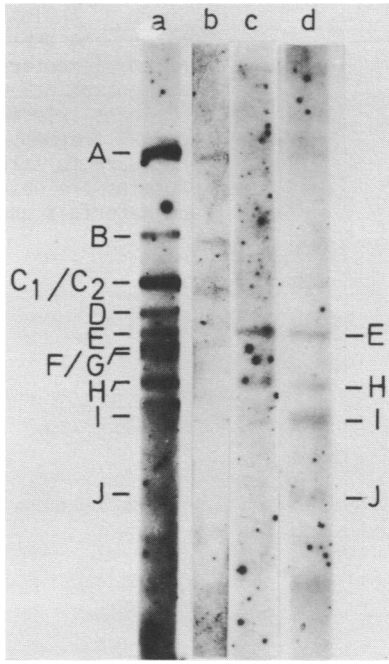

Figure 8. Analysis of RNase A-protected nuclear RNA sequences complementary to AluI-cleaved EcoRI-F fragments electrophoretically transferred to DBM paper. Lane a; blot strip exposed to nick-translated ad2 DNA. Lane b; micrococcal nuclease-degraded hnRNA. 30S particle RNA samples exposed to 2 and 20 µg/ml of RNase are shown in lanes c and d. Details can be found in the legend of figure 6 and in Materials and Methods.

absent in the controls using pBR322 for selection. The label is under these conditions confined to a small oligonucleotide covalently linked to the protein (see Materials and Methods).

DISCUSSION

This paper attempts to characterize the RNP structure of a single mRNA precursor species, transcribed from early region 2 of the ad2 genome. Technically, these experiments are difficult but it appears that the end-labelling method of protected RNA fragments will not artifactually identify underrepresented RNA sequences during the hybridization reactions (Figs. 6 and 8). The possible collapse of RNP structures after treatment with high amounts of RNase (9,25) might distort the results but the accumulation of specific RNA sequences after extensive treatment with RNase speaks against his possibility. By using RNase A to remove RNA

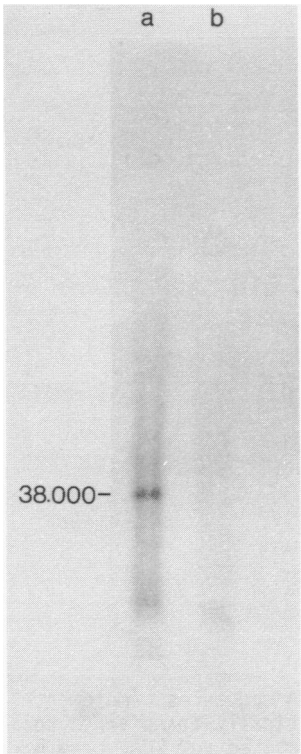

Figure 9. An 10% SDS polyacrylamide gel showing protein crosslinked to RNA complementary to the EcoRI-F fragment of Ad2 DNA. RNA protein complexes selected on a) the EcoRI-F fragment and b) pBR322. The procedure to select and analyze the proteins are described in the text and Materials and Methods.

sequences not involved in an RNP configuration, fragments of 30 bases or more appear to be protected by proteins. These protected fragments appear to contain intron sequences corresponding to the genomic EcoRI-F fragment irrespective whether they are derived from either large hnRNP complexes or 30S particles. One protected region can be found at or close to the first leader-splice junction encompassing less than 100 bases on the intron side. The other region resides in the middle of the intron. The second intron resides in the EcoRI-B fragment and could not be analyzed in detail since we did not have the DNA sequence.

It is of interest that the RNA sequences derived from the AluI-E and H (intron-specific) fragments are less strongly protected against RNase A digestion as opposed to RNA sequences from AluI-I and J (splice junction) fragments (Fig. 8). This finding may suggest that RNP structures exist in at least two different configurations as previously has been suggested by isolation of subgroups of 30S particles with different protein composition (26). If so, hnRNP molecules derived from the early region 3 located in

the EcoRI-D fragment may belong to the category exhibiting a low degree of RNase A protection (Fig. 6).

The protected RNA fragments do not correspond to long stretches of purine residues in the RNA as deduced from the DNA sequence (22). The preferential hybridization to the EcoRI-F fragment after extensive RNase treatment of the RNP may be due to the fact that the DBP precursor mRNA is more abundant in the nucleus than other species of early precursor mRNA (20 and Fig. 3).

Our results suggest that the RNA sequences associated with the 30S particles are similar to those present in hnRNP complexes. Although extensive attempts to generate 30S particles from large HnRNP complexes using low amounts of RNase A have failed in our hands, the RNA sequences protected in 30S particles appear to be the same as those present in hnRNP complexes. The 30S particles and the hnRNP complexes may therefore contain similar entities, possibly corresponding to the defined beads observed in the electron microscope (4). It is unlikely that both structures would be generated by non-specific association of basic proteins to the same RNA sequences. We would therefore like to conclude that at least some of the hnRNP proteins are nonrandomly distributed within the precursor mRNA transcript. It is not yet resolved whether these RNPs are located in both introns and exons, but at least in the DBP precursor RNA two intron-specific sites are preferentially covered by proteins. It also appears established that the 30S particles are integral parts of the large hnRNP complexes, since a similar set of RNA sequences are protected in both structures.

Using the in vivo crosslinking of RNA to protein (23,24), we also demonstrated that one of the two major hnRNP protein (molecular weight 38,000 daltons) possibly corresponding to one of the C proteins (19) is associated with the precursor mRNA for the adenovirus DBP. Since intact RNA molecules were selected on the EcoRI-F fragment we cannot conclude that this polypeptide is preferentially associated with the protected RNA sequences within the intron or at the splice junction although this appears likely.

The approach used in this study may be useful to identify the location of protein on RNA sequences in other transcription units.

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