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Abundant Nuclear Ribonucleoprotein Form of CAD RNA

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Transcripts of the CAD gene in Syrian hamster cells are as abundant in the nucleus as in the cytoplasm. This was shown by in situ hybridization of whole cells and by solution and blot hybridization of subcellular fractions. Similar results were obtained both for wild-type cells and for a mutant containing amplified CAD genes in which the level of CAD RNA is 150-fold greater. CAD nuclear RNA is indistinguishable from mature mRNA by gel electrophoresis and blot hybridization. Discrete higher-molecular-weight precursors are undetectable, although the persistence of a short length of intervening sequence in the otherwise fully processed RNA is not excluded. CAD RNA is released from nuclei by sonication in physiological conditions in a ribonucleoprotein form that sediments as a broad peak at about 200S in a sucrose gradient. CAD sequences extracted from nuclei by treatment with EDTA and RNase are found in the 30S particles previously described.

Previous studies of large nuclear ribonucleoproteins (RNPs) containing mRNA and precursors of mRNA have been hampered by the heterogeneity of the material. Any one molecular species of RNA is generally present at a level of less than 1% of the total. Biochemical information about large nuclear RNPs is therefore limited to bulk properties, for the most part derived from analysis of a breakdown product of the RNP that sediments at about 30S in a sucrose gradient. This 30S complex, made up of a fragment of RNA and a set of basic proteins, is regarded as a subunit of the native structure (12, 13, 22).

In the studies reported here, we have investigated cells containing amplified genes as a source of an abundant large nuclear RNP, with a view to the possible isolation and analysis of homogeneous material. We have used a mutant, simian virus 40 (SV40)-transformed Syrian hamster cell line in which the gene for a multifunctional enzyme abbreviated CAD (for carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydro-orotase) is amplified about 200-fold (7, 11, 16, 27). The levels of CAD mRNA and protein show a corresponding increase, CAD mRNA making up about 1% of the cytoplasmic polyadenylated RNA (16, 27). One of our first objectives was to determine whether CAD sequences are similarly enriched in nuclear RNA.

A further feature of the CAD gene pertinent to studies of nuclear RNP is its size and complexity. The structure of the gene indicates that the primary transcript is 25 kilobases (kb) in length and contains 37 or more intervening sequences (17). Hybridization analysis reveals a mature mRNA of about 7.9 kb (16, 27). The great length of these molecules heightens interest in the mechanism of their packaging, processing, and transport and at the same time poses difficulties for biochemical studies, for example, the problem of instability due to a large target size for RNase.

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MATERIALS AND METHODS

Cells. PALA-sensitive ($PALA^s$) cell line SV28 and PALA-resistant ($PALA^r$) cell line 165-28, derived from SV28 (11), were grown in culture as described previously (24).

Nuclear and cytoplasmic fractions. (i) Procedure 1. Approximately 10^8 cells scraped from subconfluent plates were washed three times with ice-cold 137 mM NaCl–8 mM Na_2HPO_4–1.5 mM KH_2PO_4–2.5 mM KCl, allowed to swell for 10 min in 2 ml of 10 mM Tris-hydrochloride –3 mM $MgCl_2$ (pH 7.5) (TM), and lysed with 10 strokes of a Dounce homogenizer (B pestle) at 4°C. Nuclei were pelleted and washed, once with 1 ml of TM containing 0.5% Triton X-100 and once with 1 ml of TM. The postnuclear supernatant and detergent wash were combined and designated the cytoplasmic fraction.

(ii) Procedure 2. Subconfluent plates were washed with 125 mM KCl–30 mM Tris-hydrochloride (pH 7.5)–5 mM magnesium acetate–1 mM 2-mercaptoethanol–2 mM ribonucleoside vanadyl complex (2)–0.15 mM spermine–0.05 mM spermidine at 4°C, and cells scraped from the plates were washed twice with the same buffer. Approximately 10^8 cells were allowed to swell for 10 min in 2.5 ml of swelling buffer (same as wash buffer except the KCl concentration was 10 mM), lysed with 20 strokes of a Dounce homogenizer (B pestle), overlaid on an equal volume of swelling buffer containing 25% glycerol, and centrifuged for 5 min at 400 × *g* and 4°C. The upper layer of the supernatant, which contained 90% of the CAD sequences released by lysis, was designated the cytoplasmic fraction. The nuclear pellet was washed once with 2 ml of swelling buffer–25% glycerol–0.5% Triton X-100 and once with 2 ml of swelling buffer.

Nuclear and cytoplasmic RNA. Nuclei were resuspended in 2 ml of TM and lysed by adding 2 ml of 20 mM Tris-hydrochloride (pH 7.5)–0.7 M NaCl–10 mM EDTA–2% sodium dodecyl sulfate–40% (vol/vol) formamide. Four milliliters of phenol-chloroform-isoamyl alcohol (50:48:2) was added, and the DNA was sheared by passing the mixture several times through a 22-gauge syringe needle. The mixture was centrifuged at 8,000 rpm for 20 min, and the separated phases were reextracted. Nuclear RNA was precipitated from the combined aqueous phases by adding 2 volumes of ethanol. Alternatively, nuclei resuspended in TM were treated with

10 volumes of guanidinium thiocyanate solution, and RNA was recovered as described below for total cellular RNA. The cytoplasmic fraction was extracted three times with phenol-chloroform-isoamyl alcohol (50:48:2), the organic phases were reextracted with 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5) (TE), the combined aqueous phases were made 0.3 M in sodium acetate (pH 5.5), and cytoplasmic RNA was precipitated by adding 2 volumes of ethanol.

Total cellular RNA. Cells were extracted with guanidinium thiocyanate, and RNA was purified by sedimentation through cesium chloride by the method of Chirgwin et al. (6), with the following modifications. Growth medium was removed from a monolayer of cells (100-mm plate) and replaced with 1 ml of guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl [pH 7.0]; Sigma Antifoam A [1 drop per 10 ml of solution] and 2-mercaptoethanol [0.1 M] were added before use). The homogeneous solution was pipetted several times to reduce its viscosity, layered in cellulose nitrate ultracentrifuge tubes (0.5 by 2 in.) half filled with 5.7 M CsCl–0.1 M EDTA (pH 7.0)–0.2% diethyl pyrocarbonate, and centrifuged in a Beckman SW50 rotor at 35,000 rpm for 15 h at 15°C. After centrifugation the supernatant was removed, leaving the RNA pellet and layer of CsCl solution, and the walls of the tubes were washed with small portions of guanidinium thiocyanate solution. Finally, the CsCl solution was decanted, the bottom 1 cm of the tubes was cut off, and the pellet was rinsed once with 70% ethanol. The RNA was resuspended in TE, made 0.3 M in sodium acetate (pH 5.5), and precipitated with 2.5 volumes of ethanol. Typically, one confluent plate (10^7 cells) yielded 200 μg of RNA, free of DNA as shown by gel electrophoresis and by fluorimetric titration with 3,5-diaminobenzoic acid (25). Total RNA was stored either as an ethanol precipitate or in 50% ethanol solution at −20°C.

^{32}P-labeled CAD DNA. $pCAD_{41}$, a recombinant pBR322 plasmid containing a 2.3-kb insert of CAD cDNA, was prepared as described previously (27) and labeled with ^{32}P by nick translation (21). This DNA was converted to a single-stranded form by a version of the procedure of Berk and Sharp (3). Nick-translated $pCAD_{41}$ (1 μg) was mixed with 70 mg of total RNA from PALA^r 165-28 cells, phenol extracted, and ethanol precipitated. The pellet was dissolved in 0.8 ml of 0.2 M PIPES (piperazine-*N*-*N*′-bis 2-ethanesulfonic acid; pH 6.4)–2 M NaCl–0.5 M EDTA. Deionized formamide (3.2 ml) was added, and the solution was incubated at 75°C for 10 min and then hybridized at 55°C for 24 h. The hybridization solution was mixed rapidly with 9.5 volumes of ice-cold 20 mM sodium acetate (pH 4.6)–0.28 M NaCl–0.5 mM zinc acetate. Denatured, sonicated calf thymus DNA was added to a final concentration of 20 μg/ml, and the mixture was digested with 100 U of S1 nuclease per ml at 45°C for 30 min. After phenol extraction and ethanol precipitation, the nucleic acids were dissolved in 0.9 ml of TE, 0.1 ml of 1 M NaOH was added, and the mixture was incubated at 37°C for 12 h to hydrolyze the RNA. The solution was then neutralized with 1 M HCl, and the DNA was precipitated with ethanol and resuspended in TE. Single-stranded CAD DNA probe was obtained in 70 to 80% yield.

Hybridization of CAD RNA with single-stranded CAD DNA. Hybridization was carried out at 55°C in a total volume of 20 μl containing 20 mM PIPES–0.2 M NaCl–0.1 mM EDTA (pH 6.4) sealed in a glass capillary. Typically, 1 ng of $pCAD_{41}$ (10^7 cpm/μg) was hybridized with 1 to 40 μg of RNA for 24 h. The hybridization solution was then mixed rapidly with 30 μl of ice-cold 60 mM sodium acetate–0.25 M NaCl–0.5 mM zinc acetate containing 20 μg of denatured sonicated calf thymus DNA per ml as carrier and digested with 100 U of S1 nuclease per ml for 30 min at 45°C. Samples (20 μl) were spotted onto Whatman 3MM filter paper, precipitated with 5% trichloroacetic acid, and counted.

S1 nuclease mapping of CAD RNA. RNA samples were hybridized and digested with S1 nuclease as described above for hybridization with single-stranded CAD DNA, except that $pCAD_{41}$ DNA digested with *Ava*I and ^{32}P-labeled with polynucleotide kinase was used. A 340-residue CAD DNA segment produced by hybridization and S1 nuclease digestion was fractionated in a 7 M urea-containing 4.5% polyacrylamide gel and quantitated by autoradiography and densitometry.

Gel electrophoresis and blot hybridization of RNA. RNA samples were denatured in glyoxal, fractionated in 0.8% agarose gels, and transferred to diazobenzyloxymethyl-paper as described previously (28). Hybridization of ^{32}P-labeled nick-translated $pCAD_{41}$ DNA to RNA fragments (immobilized on paper) was in the presence of 10% dextran sulfate (27, 28).

In situ hybridization. Cells were fixed and hybridized as described by Brahic and Haase (4). After photographic development, slides were stained with hematoxylin-eosin to reveal nuclei and cytoplasm. $pCAD_{41}$ was used for detection of CAD sequences. pMM_{26}, a plasmid containing mouse mitochondrial DNA (5), was a gift from D. Wallace. The plasmid DNAs were nick translated in the presence of [^{125}I]dCTP (>1,000 Ci/mmol; 50 to 60 μCi/μg of DNA), and their size was reduced to 50 to 100 base pairs as described previously (4).

Nuclear RNP. Nuclei from 10^8 cells, prepared as described above, were suspended in 1 ml of 10 mM Tris-hydrochloride (pH 8.0)–100 mM NaCl–2 mM $MgCl_2$–1 mM 2-mercaptoethanol–0.15 mM spermine–0.05 mM spermidine–10 mM ribonucleoside vanadyl complex (2)–100 U of placental RNase inhibitor (Amersham Corp.) per ml and sonicated at the maximum power setting of a Kontes micro-ultrasonic cell disrupter for 20 s at 4°C. Bacterial tRNA (2 mg) was added, to adsorb basic proteins (9), and the mixture was centrifuged for 1 min (Eppendorf microcentrifuge). The supernatant was applied to a 15 to 45% sucrose gradient in 10 mM Tris-hydrochloride–100 mM NaCl–2 mM $MgCl_2$–2 mM ribonucleoside vanadyl complex and centrifuged in a Beckman SW41 rotor for 90 min at 40,000 rpm and 4°C. RNA was recovered from gradient fractions by the addition of sodium dodecyl sulfate to 0.5%, treatment with proteinase K (200 μg/ml) for 2 h at 37°C, extraction with phenol, and precipitation with ethanol.

EDTA-treated nuclear RNP. Nuclei from 10^8 cells, prepared as described above, were suspended in 3 ml of TM, digested with 60 U of RNase-free DNase I for 5 min at 25°C, centrifuged at 7,000 rpm for 5 min at 4°C, resuspended in 5 ml of TE, and dispersed by passage through a Pasteur pipette. Bacterial tRNA was added to 1.2 mg/ml, and chromatin was pelleted by centrifugation at 7,000 rpm for 5 min at 4°C. The supernatant contained 85% of the total nuclear CAD RNA sequences.

RNase digestion of RNPs. The volume and TE and tRNA concentrations of the cytoplasmic fraction were adjusted to match those of the EDTA-treated nuclear RNP fraction. Samples (0.5 ml) from both fractions were digested with increasing levels of DNase-free RNase (10^{-2} to 10^{-5} μg/ml for 5 min at 25°C). Digestion was stopped by adding ribonucleoside vanadyl complex (2) to 2 mM. One-milliliter sam-

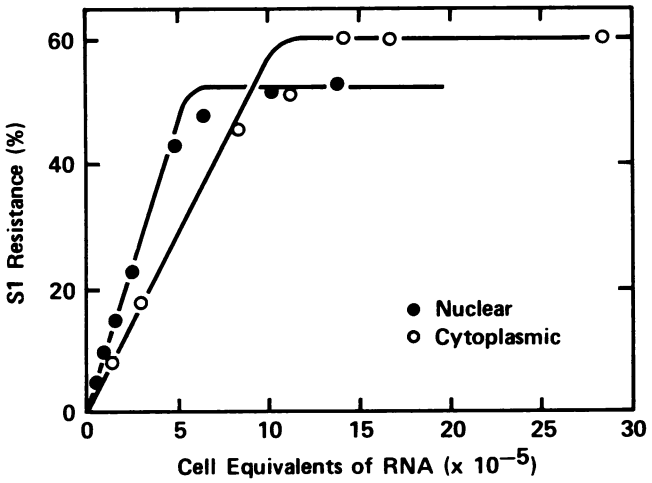

FIG. 1. Level of CAD sequences in nuclear and cytoplasmic RNA from PALA^r 165-28 Syrian hamster cells. CAD sequences were detected by hybridization with single-stranded ^{32}P-CAD DNA, S1 nuclease digestion, and trichloroacetic acid precipitation. The percent trichloroacetic acid-insoluble radioactivity was determined as a function of the amount of RNA hybridized with a fixed amount of DNA. The results are corrected for 15% trichloroacetic acid-insoluble radioactivity in the absence of added RNA, due to foldback DNA (nondenaturable product of nick translation).

ples were loaded on 15 to 30% sucrose gradients in TE and centrifuged in a Beckman SW41 rotor at 24,000 rpm for 17 h at 4°C. RNA was extracted from gradient fractions as described above for nuclear RNP.

RESULTS

Abundance of CAD RNA in the nucleus. The amount of CAD RNA in nuclear and cytoplasmic fractions from SV40-transformed Syrian hamster cells was measured by hybridization with single-stranded, ^{32}P-labeled CAD DNA from a cDNA clone. Unreacted DNA was digested with S1 nuclease, and the radioactivity protected in hybrids was determined. The degree of protection was proportional to the amount of RNA added up to the point at which the single-stranded DNA was saturated (Fig. 1). The quantity of CAD RNA sequences could be calculated from these data (slopes of lines in Fig. 1) and the known specific activity of the labeled DNA. The results (Table 1) showed two to three

TABLE 1. Comparable abundance of CAD RNA in nuclear and cytoplasmic fractions of Syrian hamster cells[a]

Expt	No. of cells (10^8)	CAD RNA sequences (μg)		CAD RNA copies per cell		Copy ratio (nucleus/ cytoplasm)
		Nucleus	Cytoplasm	Nucleus	Cytoplasm	
1	3.3	0.54	0.28	380	200	1.9
2	6.0	ND	0.56		220	
3	1.0	0.19	0.10	440	230	1.9
4	8.0	0.10	0.04	290	120	2.5
5	1.1	0.10	0.04	210	80	2.6
6	1.0	0.14	0.07	320	160	2.0
7	1.0	0.15	1.07	350	150	2.3

[a] Levels of CAD RNA were determined for nuclear and cytoplasmic fractions from 165-28 cells as described in the legend to Fig. 1. Each value represents an average of three to five determinations. The average CAD RNA copies per cell were: nucleus, 330; cytoplasm, 170. The average copy ratio of nucleus to cytoplasm was 2.0. ND, Not determined.

TABLE 2. Comparable abundance of CAD RNA in nuclear and cytoplasmic fractions of various cell lines[a]

Cell line	Copies of CAD RNA per cell		
	Total	Nuclear	Cytoplasmic
165-28	700	331	164
SV28	ND	7.5	3.5
B8-4	ND	201	69

[a] Levels of CAD RNA in nuclear and cytoplasmic fractions were determined as described in the legend to Fig. 1. Data for 165-28 (SV40-transformed, amplied CAD genes), SV28 (SV40-transformed parent of 165-28), and B8-4 (nontransformed, amplified CAD genes) cells represent averages from seven, two, and one preparations, respectively. Total CAD RNA was determined by similar analysis of RNA extracted from intact cells with guanidinium thiocyanate, and the value given is the average of two preparations. The yields of cellular RNA obtained by the guanidinium thiocyanate procedure were 1.9 and 2.0 mg/10^8 cells for these preparations. ND, Not determined.

times as many copies of CAD RNA in the nuclear as in the cytoplasmic fraction. Although the recovery of CAD RNA in the combined fractions varied from 41 to 95% (based on data in Table 1 and total CAD RNA from Table 2), the proportion in the nuclear fraction remained fairly constant (66 to 72%).

A similar abundance of CAD RNA in nuclear fractions was found for the SV40-transformed Syrian hamster cells from which the line containing amplified CAD genes was derived and for nontransformed cells containing amplified CAD genes (Table 2). The total amount of CAD RNA per cell corresponds to 0.7% of the polyadenylated RNA (Table 2 [assuming 2% polyadenylated RNA]). Taking into account

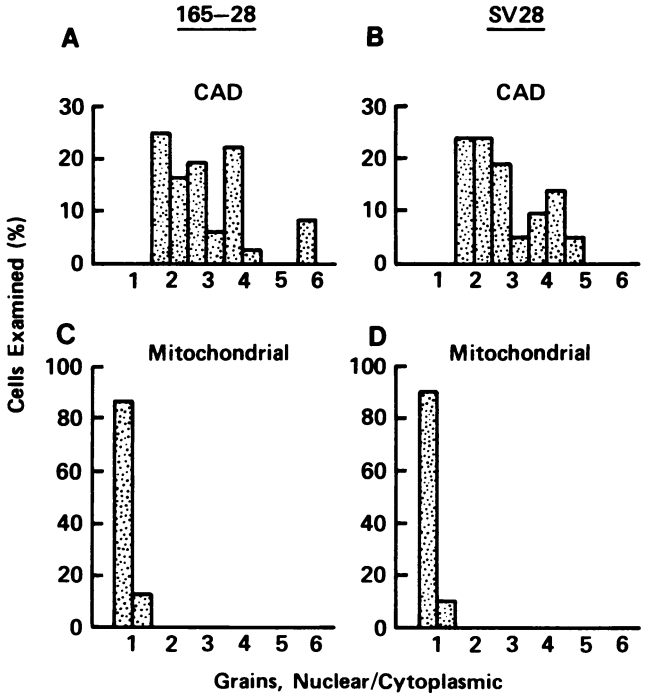

FIG. 2. CAD RNA in nucleus and cytoplasm revealed by in *situ* hybridization. PALA^r 165-28 cells (containing amplified CAD genes) and SV28 cells (parental line) were fixed and hybridized with ^{125}I-CAD and ^{125}I-mitochondrial DNA. Slides of 165-28 cells were exposed for 4 days, and those of SV28 cells were exposed for 3 weeks. Grain counts of 60 (A), 44 (B), 53 (C), and 35 (D) cells were used in constructing the histograms. There were on the average 30 to 40 grains over each cell.

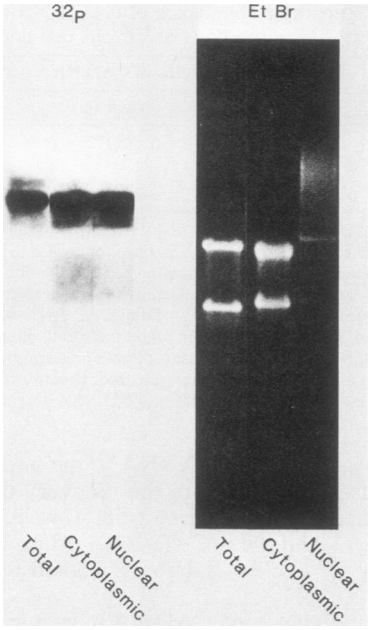

FIG. 3. Size of CAD RNA from nucleus and cytoplasm of PALA^r 165-28 cells. RNA was fractionated by gel electrophoresis, transferred to DBM paper, and hybridized with ^{32}P-labeled CAD cDNA. Right: photograph of the ethidium bromide-stained gel; left: autoradiogram of paper hybridized with ^{32}P-DNA.

the uncertainties associated with hybridization analysis, we conclude that there is as much or more CAD RNA in nuclear as in cytoplasmic fractions and further that this phenomenon is not restricted to transformed cells or to mutants containing amplified genes.

The abundance of CAD RNA in nuclear fractions was corroborated by in situ hybridization of whole cells with ^{125}I-labeled CAD cDNA. The ratio of grains over the nucleus to grains over the surrounding cytoplasm was determined, and an average value of about 3 was obtained (Fig. 2). This was in contrast with an average ratio of about 0.8 for mitochondrial sequences, believed to be restricted to the cytoplasm. The occurrence of any grains at all over the nucleus in experiments on mitochondrial sequence is presumably due to the layers of cytoplasm above and below the nucleus. When the ratio of grains over nucleus and cytoplasm found for CAD sequences is corrected for this effect, a value of 1.2 is obtained. Similar results were obtained for mutant cells containing amplified CAD genes and for wild-type cells. We conclude that the high level of CAD sequences in RNA extracted from isolated nuclei reflects the situation in vivo.

Similarity of CAD nuclear RNA to mature mRNA. On gel electrophoresis and blot hybridization, CAD nuclear and cytoplasmic RNA were indistinguishable (Fig. 3, left panel). In both cases, major and minor species of 7.9 and 10.2 kb, previously identified with CAD mRNA and shown to differ only in length of the 3′-untranslated region (17), were observed. The intensities of the bands from nuclear and cytoplasmic fractions were comparable, as expected from the quantitative analysis described above. No discrete species of higher molecular weight could be detected, although longer exposure of the autoradiogram revealed diffuse intensity extending towards the top of the gel. This lack of evidence for discrete larger precursors of CAD mRNA may be due to the large number of intervening sequences and resulting multiplicity of processing intermediates. Material of lower molecular weight than 7.9 kb was due to nucleolytic degradation, as shown by its absence from total cellular RNA extracted in conditions believed to rigorously exclude nuclease action (Fig. 4).

The apparent identity of CAD nuclear RNA with mature mRNA raises the question of whether it is a contaminant from the cytoplasm. The low level of 18S and 28S ribosomal RNAs in the nuclear fraction (Fig. 3, right panel) argues against such contamination. Since a large proportion of total cellular CAD RNA but only a trace of the ribosomal RNA is found in the nuclear fraction, the CAD RNA would have to be selectively trapped if it were derived from the cytoplasm. Further evidence for a nuclear origin of the CAD RNA in the nuclear fraction comes from studies of nuclear RNPs, described below.

CAD nuclear RNP. CAD RNP was released from nuclei by sonication; chromatin and other insoluble material was removed by centrifugation; and the clear supernatant, containing 80 to 90% of nuclear CAD sequences, was applied to a sucrose gradient. Gel electrophoresis and blot hybridization revealed a peak of CAD RNA sedimenting at about 200S in the gradient (Fig. 5). In some experiments, full-length 7.9-kb material was detected in the 200S peak, but in most cases only fragments of CAD RNA were observed. All sizes of fragment were found in the same region of the gradient, indicative of their complexation with one another or with additional components. The complexes were somewhat heterogeneous, inasmuch as the width of the 200S peak was two to three times that of a viral marker run in a parallel gradient.

CAD nuclear RNP could be distinguished from polysomes and other material of cytoplasmic origin in several ways. First, the 200S CAD peak was devoid of 18S ribosomal RNA, which remained near the top of the sucrose gradient (Fig. 5; quantitation by densitometry of autoradiograms showed that in the 200S region of the gradient there was less 18S rRNA than CAD RNA by at least an order of magni-

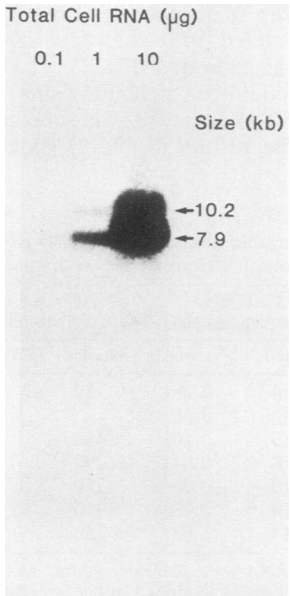

FIG. 4. CAD RNA extracted with minimal RNase degradation. Total RNA from PALA^r 165-28 cells was extracted with guanidinium thiocyanate and analyzed as in Fig. 2.

tude). Second, a cytoplasmic fraction, prepared as described above and then sonicated and analyzed in a sucrose gradient in the same manner as the nuclear fraction, gave markedly different results (Fig. 6). There was no peak of CAD RNA but rather a nearly featureless distribution across the gradient, and 18S rRNA was broadly distributed as well.

Finally, CAD nuclear RNP was characterized by conversion to a form previously described. High-molecular-weight nuclear RNPs are broken down with EDTA and RNase to 30S particles (30). In contrast, the same treatment of cytoplasmic RNP results in complete degradation of the RNA (19). When CAD RNP was extracted from nuclei in the presence of EDTA, digested with pancreatic RNase, and analyzed in a sucrose gradient, most CAD sequences were found in a peak with a sedimentation coefficient of approximately 30S (Fig. 7). Upon similar treatment of cytoplasmic material, most CAD sequences were found near the top of the gradient. Even on digestion with 10-fold less RNase, cytoplasmic CAD RNP was completely degraded. This difference in RNase sensitivity between CAD nuclear and cytoplasmic RNPs provides further evidence for the lack of cross contamination between the two preparations.

DISCUSSION

The abundance of CAD nuclear RNA and its apparent identity with mature mRNA raise the possibility of cytoplasmic contamination. Although some degree of such contamination is unavoidable, it seems unlikely to complicate the results presented here, for the following reasons: (i) the abundance of CAD sequences found in isolated nuclei conforms with the distribution of these sequences in vivo, revealed by in situ hybridization; (ii) nuclear RNA preparations contained very little 18S or 28S ribosomal RNA; (iii) the CAD RNA and 18S rRNA components of a nuclear RNP preparation were resolved in a sucrose gradient; and (iv) CAD nuclear RNP was converted by RNase digestion to 30S particles, whereas CAD sequences in cytoplasmic fractions were fully degraded.

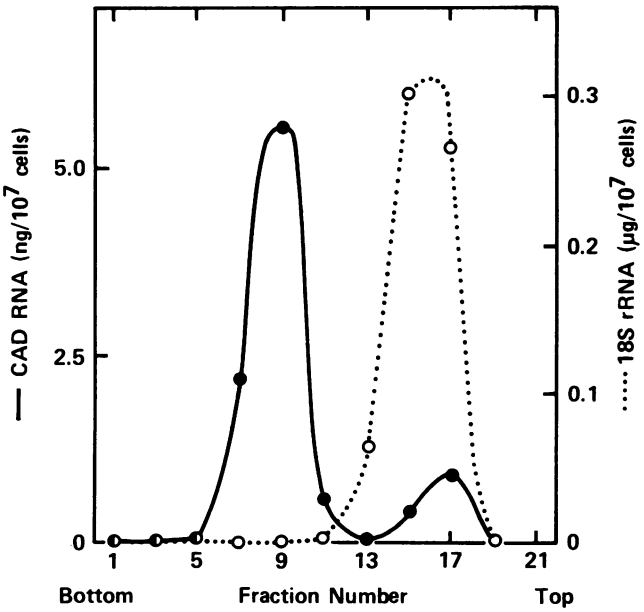

FIG. 5. RNP form (200S) of CAD RNA. Nuclear RNPs were prepared as described in the text and sedimented in a sucrose gradient. The gradient was collected in 20 fractions, starting from the bottom. The RNA was extracted and analyzed by gel electrophoresis and blot hybridization with $pCAD_{41}$ (————) and with 18S ribosomal cDNA (. . .) as described in the text. The amounts of specific RNAs in each fraction were estimated by densitometry of the autoradiograms, using as standards samples of total RNA in which the amounts of the specific RNAs were determined by solution hybridization. The peak of a 130S marker of tomato bushy stunt virus was in fraction 12 of a parallel gradient.

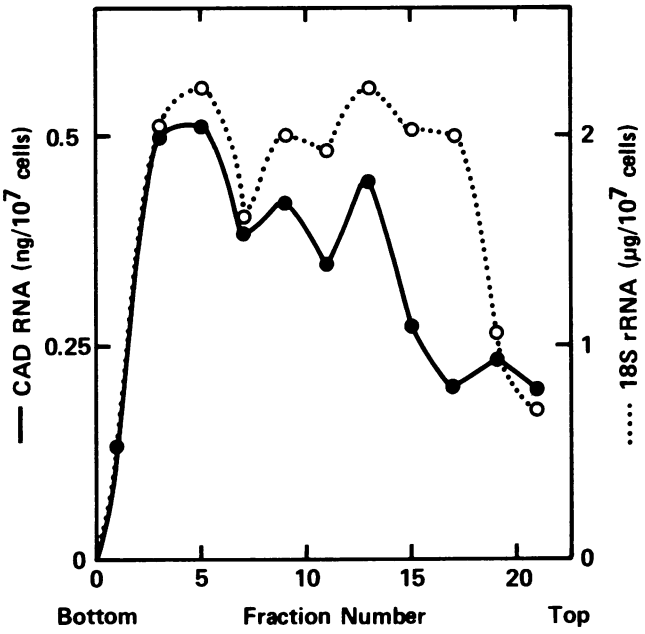

FIG. 6. Cytoplasmic RNP. A cytoplasmic fraction was prepared as described in the text and sedimented in a sucrose gradient as in Fig. 5. RNA was extracted and analyzed by dot blot hybridization and densitometry. The peak of a 130S marker of tomato bushy stunt virus was in fraction 12 of a parallel gradient.

Despite the similarity in size of CAD nuclear and cytoplasmic RNA, the persistence of a short length of intervening sequence in the nuclear species is not excluded. It also remains to be determined, for example, by pulse-chase experiments, whether most mRNA molecules in the nucleus are destined for transport to the cytoplasm. If these questions are dealt with, and it is established that CAD nuclear RNA is mostly in the form of fully processed molecules awaiting transport, then two conclusions may be drawn: (i) splicing is not coupled to transport of the mature RNA, and (ii) transport is the rate-limiting step in expression of the CAD gene. A relationship between splicing and transport was previously suggested on the basis of genetic analysis of SV40 (8, 26). Mutants lacking or unable to excise intervening sequences do not accumulate stable transcripts in the cytoplasm. This reveals a requirement for splicing at some stage in the maturation of RNA. It does not indicate that splicing and transport take place in a coupled or concerted process. Indeed, there are many genes that occur naturally without intervening sequences whose transcripts are nonetheless transported to the cytoplasm (1, 10, 15).

A predominance of molecules the size of fully processed mRNA in the nucleus and a paucity of larger precursors have been found for transcripts of globin and immunoglobulin (18, 20), as well as CAD genes. The conclusions drawn from studies of CAD nuclear RNA therefore may be quite general. At the same time, the caveats mentioned above apply, and of these, only the question of cytoplasmic con-

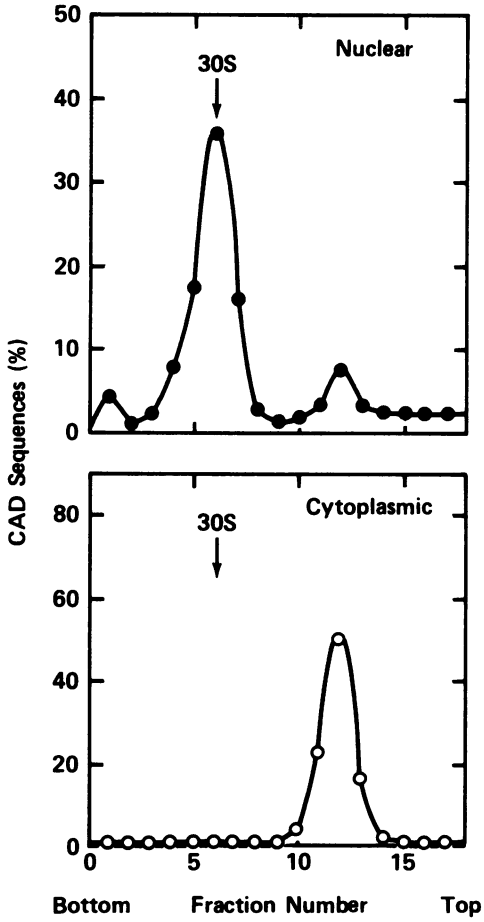

FIG. 7. Conversion of CAD nuclear RNP to 30S particles. Nuclear RNPs and a cytoplasmic fraction from PALA^r 165-28 cells were treated with EDTA, digested with RNase (10^{-4} μg/ml), and sedimented in sucrose gradients. CAD RNA was detected by hybridization with single-stranded ^{32}P-CAD cDNA. The arrow at 30S marks the position of a peak of 30S particles, prepared from nuclei of 165-28 cells pulse-labeled with [^{3}H]uridine as described previously (12, 13, 21) and analyzed in a parallel gradient.

tamination of nuclear fractions containing globin RNA has been considered (19).

The significance of the 200S form of CAD nuclear RNP is unclear. It may represent fragments of the so-called nuclear matrix, to which nuclear RNA is known to adhere (14). Alternatively, it may reflect the organization of nuclear RNA molecules in large, particulate structures, such as Balbiani ring granules (23) or perichromatin granules (29). The problem of deciding among these and additional possibilities emphasizes the need of a functional assay for large nuclear RNP preparations.

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