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The Role of Cytoplasmic Membranes in Controlling the Transport of Nuclear Messenger RNA and Initiation of Protein Synthesis

[yeast mutants/poly(A)-containing RNA/informosomes]

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ABSTRACT **Three different classes of poly(A)-containing RNA have been identified in the yeast system. They have been characterized by their kinetics of labeling, by their localization in the nuclear, membranous, and soluble cytoplasmic fractions, and by their size. The evidence indicates that the membrane-bound poly(A)-containing RNA is a product of the nuclear poly(A)-containing RNA and is the precursor of the polysomal poly(A)-containing RNA. In addition, it has been demonstrated that transport of mRNA is coupled with protein synthesis.**

Transport of messenger RNA (mRNA) via cytoplasmic membranes exists in ascites tumor cells (1, 2). Our findings indicate that polysomal mRNA precursor is bound to the cytoplasmic membranes, and imply that attachment to membranes may be a necessary step in the pathway of nuclear mRNA transport. The currently accepted, but not conclusively proven, informosomal model cannot then be applied to this system. This model stems from Spirin's (3) pioneer findings in the post-ribosomal fraction of putative mRNA in the form of ribonucleoprotein particles, and Georgiev's (4) observations of similar ribonucleoprotein in nuclear extracts. The model postulates that the mRNA of the post-ribosomal ribonucleoprotein, presumably a product of the mRNA of the nuclear ribonucleoprotein, is the polysomal mRNA precursor.

We postulate that the cytoplasmic membranes, in conjunction with the nuclear membrane, may link the chromatin with the polysomes (2). The validity of this concept rests with the answer to the question—is the membrane-bound mRNA a product of the nuclear mRNA as well as the precursor of the polysomal mRNA? Here we present evidence that answers this question affirmatively. Since these experiments have been made in yeast, this novel mechanism of mRNA transport can be applied to systems other than ascites.

MATERIALS AND METHODS

Two thermosensitive yeast (*Saccharomyces cerevisiae*) mutants, one which suppresses transport of mRNA (ts-136) (5), and the other which suppresses initiation of protein synthesis (ts-187) (6), at 36°, but both of which behave like the parental strain at 23°, were grown in synthetic complete medium (SCM) and spheroplasts were prepared (7, 8).

Cell Fractionation. The spheroplasts were harvested as explained in the figures. Cells (0.5 to 2.0 × 10^9) were resuspended in 5 ml of lytic buffer containing 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) pH 7.6, 20 mM KCl, 3 mM $MgCl_2$, and 0.01% spermidine. Triton X-100 was added to a final concentration of 0.5% and the suspension homogenized with a Teflon homogenizer for 10 strokes at high speed. Finally, it was centrifuged for 30 min at 10,000 × *g*. The supernatant represents the soluble cytoplasmic fraction. The pellet was resuspended in 5 ml of the same lytic buffer. Triton X-100 and sodium deoxychloate were added to a final concentration of 0.5% each. The detergent-treated suspension was homogenized with a Teflon homogenizer for 5 strokes at high speed and centrifuged for 30 min at 10,000 × *g*. This second supernatant represents the membranous fraction. The final pellet was resuspended in 5 ml of the same lytic buffer and represents the crude nuclear fraction.

Isolation and Characterization of Poly(A)-containing RNA. Poly(A)-containing RNA was isolated by a modification (8) of the method of Perry *et al.* (9). The determination of radioactive poly(A)-containing RNA yielded identical values when the Millipore binding technique (10), the poly(U) glass fiber filter technique (11), or the poly(U)-Sepharose method (12) were used. Because of its simplicity, the Millipore binding technique was regularly employed.

Preliminary tests were made to assess specific recognition of cellular poly(A)-containing RNA without interference of mitochondrial poly(A)-containing RNA species. Equal amounts of cellular poly(A)-containing RNA were detected in the three fractions obtained from spheroplasts cultured both in the presence and absence of ethidium bromide (15 μg/ml added to 2 × 10^7 cells per ml) at 23° and at 36°.

For the determination of the total amount of poly(A)-containing RNA, the procedure of Wilt (13) was applied. The [^{3}H]poly(U) was obtained from Schwarz/Mann, specific activity 23.6 Ci/mol of phosphorus. Radioactivities were determined by scintillation counting in Bray's solution.

Sedimentation Velocity Gradients. Gradients of 0.5–15% sucrose in a solution containing 99% dimethylsulfoxide, 1 mM sodium ethylenediamine tetraacetate, and 0.25 mM of Tris·HCl, pH 7.4, were made by a modification of the procedure of Strauss *et al.* (14). The total RNA recovered in the void volume of the poly(U)-Sepharose column and the RNA eluted from the poly(U)-Sepharose with 90% formamide solution were precipitated by addition of 0.1 volume of 1 M NaCl and 3 volumes of ethanol. The precipitates were dissolved in 50 mM Tris·HCl buffer pH 7.6 and dimethylformamide and dimethylsulfoxide added to final concentrations of 33% and 55%, respectively. The samples were heated at

Abbreviation: SCM, synthetic complete medium.

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60° for 5 min and then over-layered. The gradients were centrifuged in a Spinco SW 65 rotor at 55,000 rpm, at 25°, for 22 hr, fractionated, and the radioactivity determined by scintillation counting in Bray's solution.

RESULTS AND DISCUSSION

The nuclear poly(A)-containing RNA and the polysomal and nonpolysomal poly(A)-containing RNA of the soluble cytoplasmic and membranous fractions were characterized. The majority of the polysomes and all of the ribosomal subunits were recovered in the soluble cytoplasm‡. In the soluble cytoplasmic and membranous fractions the majority of the poly(A)-containing RNA was released by ethylenediaminetetraacetate and, hence, was associated with polysomes‡. However, membranes appeared to have more nonpolysomal mRNA (25–30%) than the soluble cytoplasm (12–19%) (not shown). In any case, since the majority of poly(A)-containing RNA was associated with polysomes, this justifies, for practical purposes, the use of the whole fraction to extract this RNA. Finally, the poly(A)-containing RNA extracted from the detergent-treated nuclei was considered to be composed of nuclear pre-mRNA and mRNA.

The kinetics of labeling of nuclear poly(A)-containing RNA and its entrance into the membrane and soluble cytoplasm are shown in Fig. 1. With ts-136 mutants cultured at 23° a linear uptake of the radioactive precursor into the nuclear poly(A)-containing RNA was obtained, and a linear entrance of the radioactive poly(A)-containing RNA into the membranes as well as into the soluble cytoplasm was observed (Fig. 1*a*). An essentially similar pattern of labeling was obtained when the amount of poly(A), instead of the poly(A)-containing RNA, was measured (Fig. 1*a*, *insert*).

These results indicate that at 23° there is rapid transport of the nuclear poly(A)-containing RNA, with its subsequent and exclusive accumulation in the soluble cytoplasmic fraction. The labeling experiments direct attention to the similarity between the nuclear and membranous fractions. It is reasonable to expect that the latter might contain "leaked" nuclear poly(A)-containing RNA, since the yeast cytoplasmic membranes are tightly bound to the nuclear membrane‡. This expectation was explored by similar labeling studies in spheroplasts maintained at 36°, since this temperature appears to suppress mRNA transport in this mutant (5). Fig. 1*b* indicates that in this condition poly(A)-containing RNA accumulates in the nuclear instead of in the soluble cytoplasmic fraction. Since small amounts of this RNA were observed in the cytoplasmic membranes, the possibility that this fraction contained "leaked" nuclear RNA could be excluded. Furthermore, the observation that membranous and soluble cytoplasmic fractions can be differentiated by the kinetics of entry of their poly(A)-containing RNA indicates that the fractions are not cross-contaminated.

Further exploration of the presence of three distinct classes of poly(A)-containing RNA was made by the determination of their respective specific activities at 23° (Fig. 2). These were measured by comparing the amount of newly synthesized poly(A)-containing RNA to the total content in each fraction. The latter was determined by hybridization with [^{3}H]poly(U) which remained RNase-resistant (13).

Fig. 2 clearly indicates that poly(A)-containing RNAs derived from the nuclear and membranous fractions have much higher specific activities than RNA derived from the soluble cytoplasmic fraction. As with the estimation of

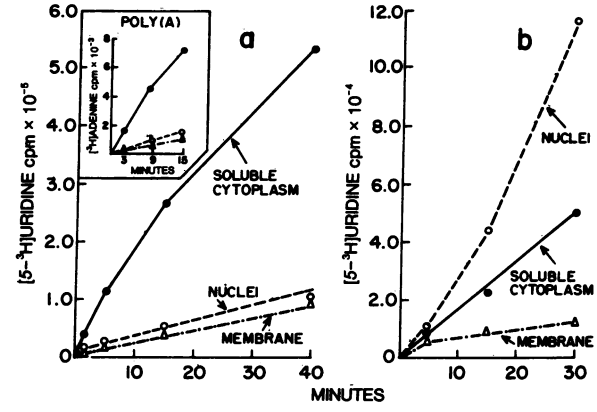

FIG. 1. Synthesis of poly(A)-containing RNA of the ts-136 mutant cultured at 23° and 36°. The spheroplast culture was divided into aliquots (4 × 10^7 cells per ml); each was labeled with the radioactive RNA precursor and, at the indicated interval, rapidly poured into a slushy 1 M D-sorbitol solution; the spheroplasts were harvested and fractionated, and the RNA was extracted (see *Materials and Methods*). The poly(A)-containing RNA was determined by the Millipore technique (10). (*a*) Kinetics of [5-^{3}H]uridine incorporation at 23°. Each 50-ml aliquot was labeled with 50 μCi of this RNA precursor. The *insert* shows the kinetics of [8-^{3}H]adenine incorporation at 23°. The spheroplasts were resuspended in SCM containing 2 mg of adenine per liter, and each 100-ml aliquot was labeled with 100 μCi of the RNA precursor. (*b*) Kinetics of [5-^{3}H]uridine incorporation at 36°. The spheroplasts were cultured in 80-ml aliquots at 36° for 1 hr. Afterwards, each aliquot was labeled with 100 μCi of the RNA precursor. (●), Soluble cytoplasm; (○), nuclei; (△), membrane.

absolute amounts (Fig. 1), similar specific activities of poly(A)-containing RNA were obtained in the nuclear and membranous fractions. Note that the labeling of the nuclear pool of poly(A)-containing RNA does not reach a steady-state condition. With the data available it is difficult to discern if such a result is obtained because there are poly(A)-containing RNA species which are synthesized at different times, because there is expansion of the nuclear pool due to cellular

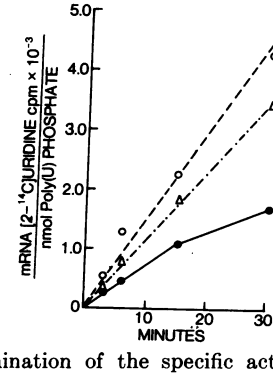

FIG. 2. Determination of the specific activity of poly(A)-containing RNA extracted from the ts-136 mutant cultured at 23°. Each 100-ml aliquot (4 × 10^7 cells per ml) labeled with 6 μCi of [2-^{14}C]uridine at the indicated intervals was collected and processed as explained in the *legend* of Fig. 1 and in *Materials and Methods*. The determination of newly synthesized poly(A)-containing RNA was made by the Millipore procedure (10), and the total amount of this RNA was measured by the [^{3}H]poly(U) hybridization technique (13). Both determinations were made in the same sample. (●), Soluble cytoplasm; (○), nuclei; and (△) membranes.

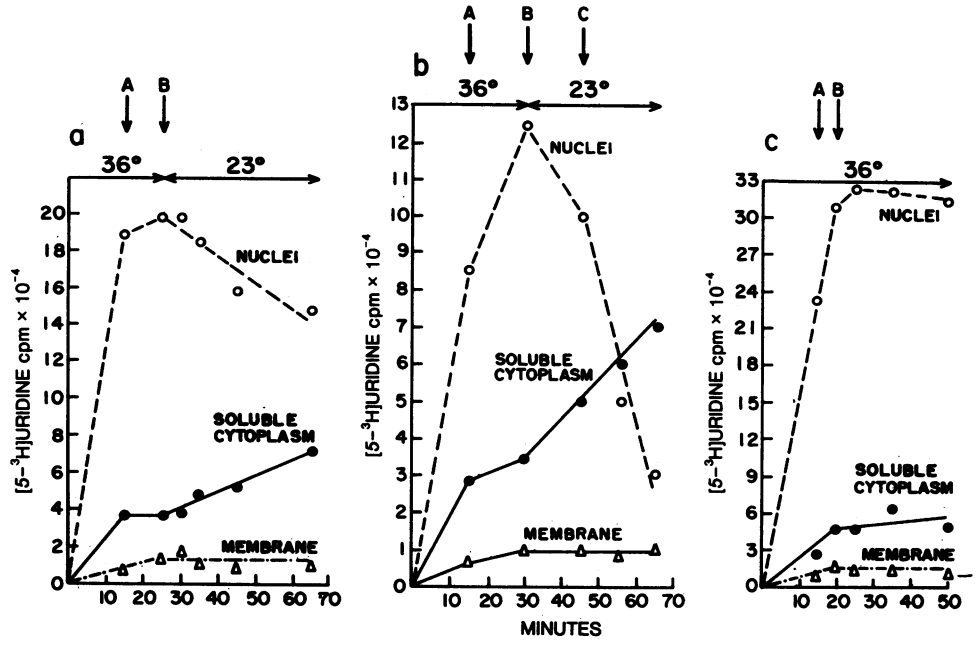

FIG. 3. Pulse-chase experiments in the ts-136 mutant. Three experiments were made. In each, 200 ml of a spheroplast suspension (4×10^7 cells per ml) was cultured at 36° for 1 hr. Then 100 μCi of [5-^{3}H]uridine was added, followed 15 min later by 200 mg of nonradioactive uridine. The spheroplasts were then harvested, rapidly resuspended in 120 ml of SCM, pre-warmed to 36°, and supplemented with 120 mg of nonradioactive uridine in the absence of glucose ("chase-medium"). Immediately after, 20-ml aliquots were taken and the rest maintained at 36°. In (*a*) and (*b*) 15 min later 400 ml of the "chase-medium" kept at 23° was added. In (*c*) 5 min later 400 ml of the "chase-medium" kept at 36° was added. In (*b*) 15 min after the temperature-shift glucose was added to 20 g/liter. At the indicated intervals 100-ml aliquots were withdrawn, the spheroplasts were harvested and fractionated, and the RNA was extracted. For determination of poly(A)-containing RNA see *legend* of Fig. 1. *Arrow A* indicates the beginning of the "chase"; *B* indicates the time at which the 400 ml of the "chase-medium" was added; and *C* indicates the time at which glucose was added. (●), Soluble cytoplasm; (○), nuclei; (△), membranes.

growth, or because there is a portion of this RNA which might turn over within the nucleus.

Since the membrane is not the site of synthesis, the high specific activity of the membrane-bound poly(A)-containing RNA indicates that there must either be a rapid turnover of this class of RNA or a quick release into the soluble cytoplasm. To discriminate between these two possibilities pulse-chase experiments were made.

As transport of poly(A)-containing RNA is rapid and as actinomycin D does not completely suppress yeast RNA synthesis, the conventional procedure for pulse-chase experiments cannot be applied to this system (15). Accordingly, use was made of the fact that the ts-136 mutant accumulates nuclear poly(A)-containing RNA at 36° (Fig. 1*b*). Furthermore, immediate inhibition of the uptake of the radioactive precursor into this RNA can be effected by addition of nonradioactive RNA precursor, followed by glucose deprivation in the medium.

Preliminary experiments established that normal transport of poly(A)-containing RNA occurred in the absence of glucose. Although in this condition only 10% of the protein and RNA synthetic machineries are in operation, no damage occurs to the system, since the addition of glucose results in a rapid recruitment of ribosomes into polysomes and an immediate onset of RNA synthesis‡.

As shown in Fig. 3, soon after the spheroplasts were transferred to the permissive temperature, transport of poly(A)-containing RNA occurred (Fig. 3*a* and *b*). Diminution of this RNA in the nuclear fraction was followed by accumulation in the soluble cytoplasmic fraction, while the amount of membrane-bound poly(A)-containing RNA was essentially constant. These observations indicate that the high specific activity of the poly(A)-containing RNA associated with the membrane is compatible with its discharge into the soluble cytoplasm and not with its rapid turnover. The data so far indicate that after the poly(A)-containing RNA is synthesized in the nucleus it is rapidly transported into the membranes and released into the soluble cytoplasm. Evidently, the membranes are an obligatory step in the pathway of mRNA transport.

The experiment also established that there is no significant degradation of nuclear poly(A)-containing RNA synthesized and retained at 36° (Fig. 3*c*). However, the fact that less than half of this RNA is recovered in the soluble cytoplasm shows that it must be digested during the process of transport. As will be shown later, yeast lacks the very large heterogeneous nuclear RNA which is typical of higher eukaryotes (15). Hence, there are two possible interpretations of this observation that loss of poly(A)-containing RNA from the nucleus greatly exceeds its gain in the soluble cytoplasm: (*1*) Not all of the poly(A)-containing RNA synthesized at 36° can be translated; (*2*) since yeast mRNA has a half-life of 25 min (16), only half of the transported poly(A)-containing RNA is recovered after this interval. The latter appears more probable, since degradation of this RNA takes place only upon transport and, as we will show later, transport does not take place without initiation of protein synthesis.

‡ L. Brañes and A. O. Pogo, to be published.

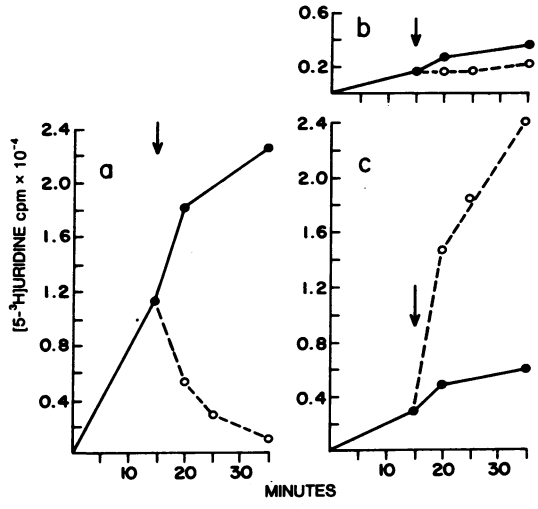

FIG. 4. Effect of inhibition of initiation of protein synthesis in the transport of mRNA (ts-187 mutant). To 300 ml of spheroplast culture (4 × 10^7 cells per ml) maintained at 23° was added 1.5 mCi of [5-³H]uridine; 15 min later 50 ml was withdrawn. The remainder was divided into two fractions. In one (150 ml) an equal volume of the SCM pre-warmed at 60° was added. To the remainder (control) an equal volume of the SCM kept at 23° was added, and 100 ml of each culture was withdrawn at the indicated intervals. Poly(A)-containing RNA was determined by the Millipore technique (10). The *arrow* indicates the time of the temperature-shift. (*a*) Soluble cytoplasm, (*b*) membrane, and (*c*) nuclei. (●), Culture maintained at 23°; (○), culture shifted to 36°.

Although glucose is not necessary for transport of poly(A)-containing RNA, addition of glucose to glucose-starved spheroplasts accelerates this process (compare Fig. 3*a* and *b*). This may be due to the increase in the rate of protein synthesis, because a rapid recruitment of ribosomes into polysomes takes place when glucose is added to a glucose-deprived medium‡. By augmenting the rate of protein synthesis, an increase can be brought about in the rate of transport in two ways: (*1*) by increasing the rate of synthesis of the thermosensitive factor(s)—supposedly a protein—necessary for transport of poly(A)-containing RNA molecules; (*2*) by increasing the number of translation complexes between the ribosomal subunits and the mRNA, resulting in augmentation of the number of membrane-bound mRNA molecules entering the polysomes. It is likely that both processes occur, but the latter has been considered in view of the influence of initiation of new polypeptide chains on the process of mRNA transport.

This is an important point in our prediction, since initiation of translation is a necessary step in the conveyance of the newly synthesized mRNA from the membrane into the polysome (1, 2). If this is correct, inhibition of transport would be expected when initiation of protein synthesis is suppressed. This has been tested by using the ts-187 mutant, which is unable to initiate synthesis of new polypeptide chains after brief incubation at 36° (6). The temperature-shift was accompanied by suppression of the transport and rapid disappearance of the poly(A)-containing RNA in the soluble cytoplasm (Fig. 4*a*). In membranes, transport of this RNA was also inhibited, while in the nucleus there was a dramatic accumulation of the newly synthesized poly(A)-containing RNA molecules. An important feature was the relative constancy of the poly(A)-containing RNA associated with the membranes. This implied that neither digestion nor release of

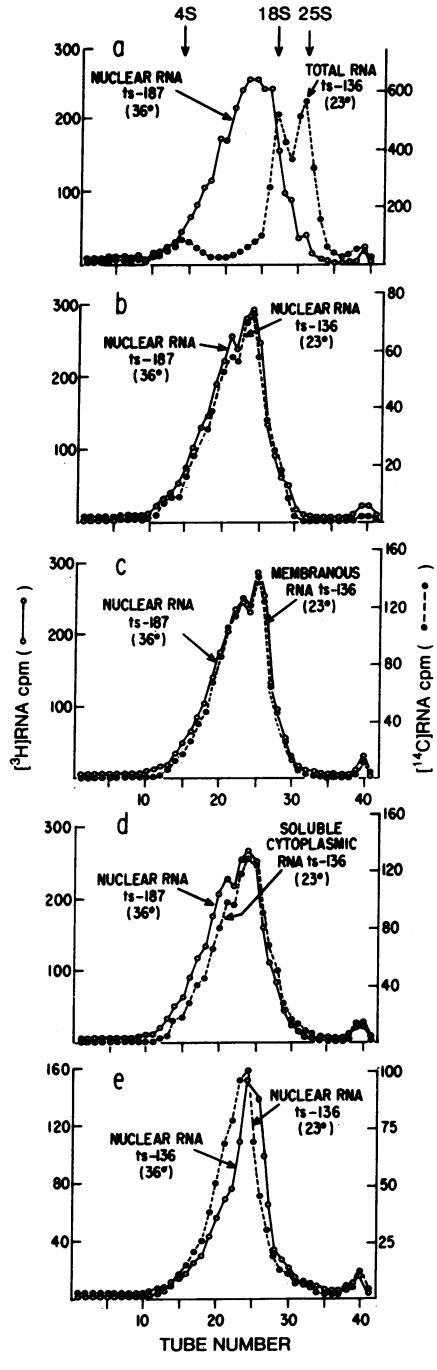

FIG. 5. Dimethylsulfoxide sucrose gradients of the poly(A)-containing RNAs absorbed to and eluted from the Sepharose column. The method of absorption to and elution from the poly(U)-Sepharose was explained elsewhere (12). The [¹⁴C]RNA and [³H]RNA were mixed, applied to the gradients, centrifuged, and fractionated, as explained in *Materials and Methods*. All the samples were counted to 3% error. In (*a*) nuclear [³H]RNA was extracted from ts-187 cultured at 36° and labeled for 15 min. The internal marker [¹⁴C]RNA was extracted from total ts-136 spheroplasts cultured at 23° and labeled for 1 hr. In (*b*), nuclear [¹⁴C]RNA; in (*c*) membranous [¹⁴C]RNA; and in (*d*) soluble cytoplasmic [¹⁴C]RNA was extracted from ts-136 cultured at 23° and labeled for 15 min. The internal marker was the same nuclear [³H]RNA as in (*a*). In (*e*) nuclear [¹⁴C]RNA was the same as in (*b*) and the nuclear [³H]RNA was extracted from ts-136 that was cultured at 36° for 1 hr and labeled for 15 min. (○), [³H]RNA; (●), [¹⁴C]RNA. In *parentheses* is the temperature at which each mutant was cultured.

the membrane-bound poly(A)-containing RNA into the soluble cytoplasmic fraction occurred in the absence of initiation of protein synthesis. It is evident that inhibition of initiation of new polypeptide chains at the membrane level produces a blockage in transport of poly(A)-containing RNA molecules. However, it can be argued that such blockage is a consequence of inhibition of protein synthesis *per se* rather than inhibition in the initiation of new polypeptide chains. The fact that glucose starvation produces a 90% inhibition in protein synthesis without inhibition in the transport of poly(A)-containing RNA does not, however, agree with this interpretation‡ (8). Furthermore, normal transport occurred in the absence of glucose (Fig. 3*a*).

To further characterize the three classes of poly(A)-containing RNA, we determined their sedimentation coefficients, using 4S, 18S, and 25S yeast rRNA as markers (Fig. 5). The coefficient of nuclear poly(A)-containing RNA of ts-187 cultured at 36° was about 11.3 S (10.4–11.4) (Fig. 5*a*). With this RNA as a marker, the sedimentation coefficient of poly(A)-containing RNA of the nuclear, membranous, and soluble cytoplasmic fractions of ts-136 cultured at 23° did not differ significantly from this value (Fig. 5*b*, *c*, and *d*). However, it appears that the sedimentation coefficient of the nuclear poly(A)-containing RNA of ts-136 synthesized at 36° was slightly higher (about 10%) than that synthesized at 23° (Fig. 5*e*). This suggests that the processing of the ts-136 pre-mRNA was affected by the temperature-shift, indicating that the thermosensitive factor(s) in this mutant may be the nuclear nucleases and/or components that are involved in the cleavage of the yeast pre-mRNA. If the nuclear poly(A)-containing RNA synthesized and retained at 36° in the ts-136 mutant is the true yeast pre-mRNA, then in yeast, as in other fungi (*Dictyostelium discoideum*) (12), the pre-mRNA is slightly larger than mRNA. The fact that no difference exists between nuclear poly(A)-containing RNA of the ts-187 strain cultured at 36° and that of the ts-136 cultured at 23° indicates that the temperature-shift in the ts-187 mutant did not affect the processing of pre-mRNA. This is consistent with the observation that, at the nonpermissive temperature, the mutant does not transport mRNA because there is a blockage in the initiation of translation (Fig. 4).

It is noteworthy that we, like McLaughlin *et al.* (17), observed that the RNA that is not bound to the poly(U)-Sepharose column contains polydisperse RNA in addition to rRNA species. However, the polydisperse RNA that does not contain poly(A) sequences appears to be restricted mainly to the membranous and nuclear fractions (not shown). With the data available, it is difficult to discern if this RNA is either mRNA without poly(A) sequences, or if it is related to viral RNA (18).

It should be emphasized that the results obtained in these experiments are based on the selection of mRNA molecules that contain poly(A) sequences. Although it has been shown that in eukaryotes the majority of mRNA molecules contain poly(A) sequences, the possibility remains that there are similar mRNA molecules that do not possess poly(A) sequences, which are thereby excluded from the determination reported here, and the fate of which remains unknown.

CONCLUSION

In summary, these experiments demonstrate that at 23° yeast nuclear poly(A)-containing RNA, presumably pre-mRNA, is rapidly processed and, as mRNA, is quickly transported, entering the polysomes through the formation of a translation complex at the level of the cytoplasmic membranes. When processing of nuclear pre-mRNA is suppressed—ts-136 cultured at 36°—there is no transport. This causes accumulation of pre-mRNA in the nuclear fraction and disappearance of mRNA from the membranous as well as the soluble cytoplasmic fraction. The data support the concept that processing of pre-mRNA is a prerequisite for transport (19). When the initiation of protein synthesis is instantly suppressed—ts-187 shifted to 36°—there is also no transport of poly(A)-containing RNA. This produces abrupt accumulation of mRNA in the nucleus with no alteration in the amount of mRNA associated with membranes, but disappearance of mRNA from the soluble cytoplasm. It is concluded that blockage of mRNA transport occurs because all the membrane binding sites are occupied as a consequence of the inhibition of release of mRNA. The fact that in the soluble cytoplasm there is no mRNA in the absence of polysomes is a further confirmation of our model of mRNA transport.

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