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A Temperature-Sensitive Mutation Affecting 28S Ribosomal RNA Production in Mammalian Cells

(somatic cell genetics/BHK 21 hamster cells/rRNA processing)

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ABSTRACT **We have characterized a temperature-sensitive (ts) mutant of the hamster cell line BHK 21 that appears to have a defect in the processing of ribosomal RNA precursors at 39°. Mutant ts 422E grows at a normal rate at 33°, but upon shift to 39° growth stops after about one cell doubling. The appearance of 28S rRNA and large ribosomal subunits in the cytoplasm of ts 422E at 39° is inhibited by about 95%, when compared to wild-type BHK cells. Production of 18S rRNA and small ribosomal subunits is unaffected. Shift-up experiments show that the defect in 28S rRNA production can be detected as early as 2–3 hr after the shift to 39°. Synthesis of the larger rRNA precursor is normal at high temperature, but the processing appears to be arrested after the formation of 32S rRNA. 32S rRNA accumulates to some extent in the nucleoli of ts 422E. ts 422E cells appear to have a single mutation, directly affecting the conversion of 32S to 28S rRNA. The reduced amount of 28S rRNA in the cytoplasm of ts 422E cells at 39° seems therefore responsible for their inability to grow at this temperature.**

In a previous publication, we described the isolation and the general properties of several temperature-sensitive (ts) mutants of the Syrian hamster cell line BHK 21/13 (1). We report now on the characterization of one of these mutants, ts 422E, whose inability to multiply at 39° seems to be due to a ts defect in the production of 28S ribosomal RNA (rRNA).

Ribosomal RNA in eukaryotic cells is synthesized as a large precursor molecule that contains the sequences of one molecule of 28S RNA (the RNA component of the large ribosomal subunit) and of one molecule of 18S RNA (the RNA component of the small ribosomal subunit), and a nonribosomal portion, destined to be discarded (2, 3). As determined mostly from studies with HeLa cells, 45S RNA precursor molecules undergo chemical modifications through the activity of methylating enzymes. Processing then occurs in a stepwise fashion within the nucleolus, resulting in the appearance of RNA intermediates and formation of ribosomal subunits that are transferred to the cytoplasm [for a review see (4)].

In the ts BHK mutant 422E, rRNA production is fully normal at low temperature, but at 39° the appearance of 28S rRNA in the cytoplasm is greatly inhibited. The mechanism responsible for this defect seems to reside in an abnormal processing of rRNA precursors arrested at the 32S stage.

MATERIALS AND METHODS

Cells. The characteristics of parental BHK 21 cells and the selection of the ts mutants have been described (1). Cells were grown at 33.5° in Dulbecco's modification of Eagle's medium, supplemented with 5 or 10% calf serum. At this temperature, wt BHK and ts 422E grow with a generation time of 16 hr. At 39°, wt BHK cells have a doubling time of 12 hr.

Cell Fractionation. Cells were fractionated into nuclear and cytoplasmic fractions essentially as described by Penman (5, 6). Cells were detached from the plate with a purified trypsin–EDTA solution, washed twice by centrifugation with cold isotonic Tris-buffered saline (pH 7.4), resuspended in hypotonic buffer (10 mM NaCl–1.5 mM $MgCl_2$–10 mM Tris·HCl, pH 7.2) at a density of about 10^7 cells per ml, and allowed to swell for 10 min at 0°. They were broken with a Dounce homogenizer and Nonidet P40 was added to a concentration of 1%. In some experiments, in order to obtain more purified nuclei a mixture of sodium deoxycholate (0.25%) and Nonidet P40 (0.25%) was used. The suspension was shaken in a Vortex Onmimixer for 30 sec and nuclei were pelleted by centrifugation at 1500 rpm (5,000 × *g*) for 4 min at 5°. The supernatant constitutes the cytoplasmic fraction.

For the preparation of nucleoli, nuclei were washed once with hypotonic buffer and lysed in high ionic strength buffer (0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris·HCl, pH 7.2). The preparation was then warmed to room temperature (24°) and incubated with 50 μg/ml of DNase for 2–3 min. The digested nuclei were layered on a 15–30% sucrose gradient in high ionic strength buffer, and centrifuged for 15 min at 18,000 rpm (38,000 × *g*) and 5° (7). The pellet constitutes the nucleolar fraction, and was resuspended in sodium dodecylsulfate (SDS) buffer (0.1 M NaCl–0.5% SDS–0.01 M Tris·HCl, pH 7.2). The supernatant represents the nucleoplasmic fraction (7); the nucleoplasmic RNA was precipitated with 2 volumes of 95% ethanol at −20°, and after centrifugation was resuspended in SDS buffer.

RNA Extraction and Fractionation. SDS was added to the cellular fractions to a concentration of 1%. RNA was either fractionated directly on linear 15–30% sucrose gradients in SDS buffer, or purified by 2–3 phenol extractions at room temperature. After extraction, RNA was precipitated with 2 volumes of 95% ethanol at −20°. SDS–sucrose gradients (16 ml) were centrifuged in an SW27 Spinco rotor at 23°. Cytoplasmic and nucleoplasmic extracts were centrifuged at 25,000 rpm for 18 hr; nuclear or nucleolar extracts were centrifuged at 23,000 rpm for 17 hr. After centrifugation, 0.5-ml fractions were collected from the bottom of the tube, and precipitated with Cl_3CCOOH on Millipore filters. Radioactivity was counted in a Beckman liquid scintillation spectrometer.

Preparation of Ribosomal Subunits. To the cytoplasmic fraction were added sodium deoxycholate to 0.5% and $MgCl_2$ to 0.07 M (8). After standing in ice for 45 min, the aggregated ribosomes were collected by centrifugation at 27,000 × *g* for

Abbreviations: ts, temperature-sensitive; SDS, sodium dodecyl sulfate; wt, wild-type.

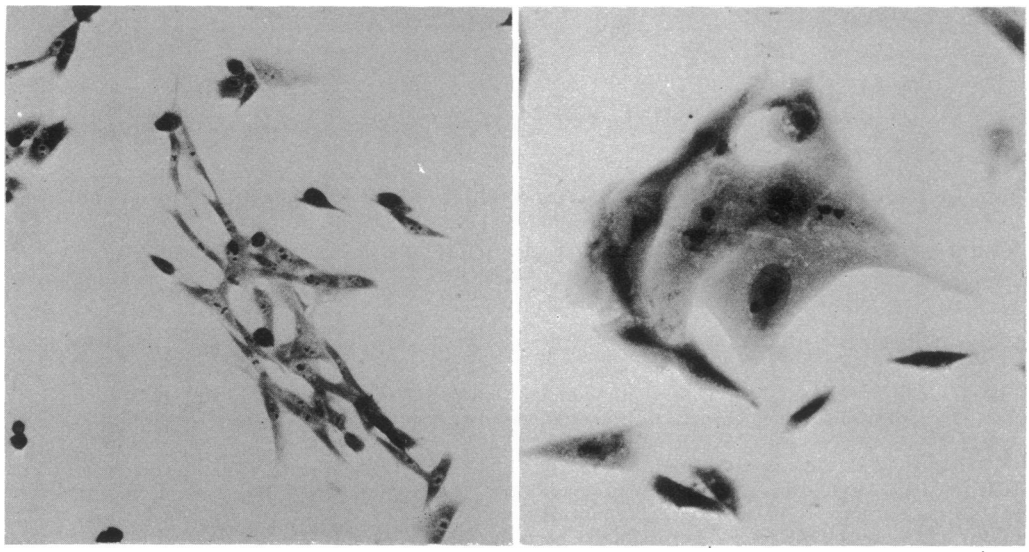

FIG. 1. Microphotographs of ts 422E cells growing at 33° (*left*) and at 39° (*right*) 5 days after shift-up. Cells were fixed in 1% gluteraldehyde and stained with Giemsa. Magnification is the same in both pictures (×320).

10 min at 5°. The pellet was resuspended in a buffer containing 0.2 M NaCl–0.01 M EDTA–0.01 M Tris·HCl, pH 7.2, and analyzed on 15–30% sucrose gradients in the same buffer by centrifugation at 24,000 rpm for 18 hr at 5° in a Spinco SW27 rotor.

Polyacrylamide Gel Electrophoresis. Gels contained 2.6% (w/v) acrylamide and 0.24% (v/v) ethylene diacrylate, and were polymerized in a buffer containing 20 mM Na acetate–1.0 mM NaEDTA–40 mM Tris-acetate, pH 7.2 (9). Electrophoresis was run at 5 mA per tube for 5 hr in the same buffer, containing in addition 0.2% SDS. Gels were sliced frozen into 1-mm slices, solubilized in 0.4 ml of a NCS (Amersham/Searle)–water mixture (9:1) at 60° for 3 hr, and counted in a toluene-based scintillation fluid.

RESULTS

The details of the isolation procedure and some of the properties of the ts BHK mutant 422E have already been described (1). Its biological properties are summarized in Table 1. When the cells are plated at 39°, their efficiency of plating is about 1×10^{-7}, while that at 33° is normal (about 50%). Thus, the reversion frequency is low, but can be increased by mutagens (1). After shift to high temperature, the cells stop growing within 24 hr, performing about one cell division, and lose viability (as judged by colony forming ability at 33°) with a half life of about 24 hr (1).

The incorporation of radioactive precursors into acid-insoluble material does not seem to be affected when the mutant is shifted to high temperature. After 48 hr at 39°, incorporation of uridine and amino acids is still about like that of wild-type cells. Thymidine incorporation is only slightly impaired.

Upon shift to 39°, the cells and their nuclei become progressively larger (Fig. 1). This phenomenon is paralleled by a conspicuous enlargement of the nucleoli, which in most cells seem to fuse into one. This behavior suggested to us that the defect responsible for the ts phenotype of these cells could have been an alteration of rRNA processing that led to an accumulation of rRNA precursors in the nucleolus.

Production of 28S and 18S rRNA. The above mentioned observation prompted an examination of the production of 28S and 18S rRNA in ts 422E cells. wt BHK and ts 422E cells were incubated at 33° and 39° for 48 hr, at which time they were labeled with [^{3}H]uridine for 2 hr at 33° or 1.5 hr at 39°. Their cytoplasmic fractions were prepared and analyzed on SDS–sucrose gradients. Fig. 2 shows that under these conditions the amounts of labeled 28S and 18S RNA appearing in the cytoplasm of wt BHK are essentially identical at 33° and 39°. At 33° the mutant behaves like wild-type cells, while at 39° the appearance of 28S RNA in the cytoplasm is greatly impaired. Production of 18S and 4S RNA appears normal.

TABLE 1. *Properties of BHK ts 422E cells*

Cells	Efficiency of plating 39°/33°	No. of divisions at 39°	Loss of viability* upon incubation at 39°	Incorporation† at 39° of radioactive precursors‡		
				dT	U	Amino acid
ts 422E	about 1×10^{-7}	1.1	50%/day	40	100	100
wt BHK	1	∞	None	100	100	100

* Defined as ability to form colonies at 33°.

† Determined as % of radioactivity incorporated into Cl_3CCOOH-insoluble material by the same number of wild-type cells.

‡ Determined 48 hr after shift to 39°, by pulsing cells for 2 hr in the presence of [^{3}H]dT, [^{3}H]U, or [^{14}C]aminoacid mixture, respectively.

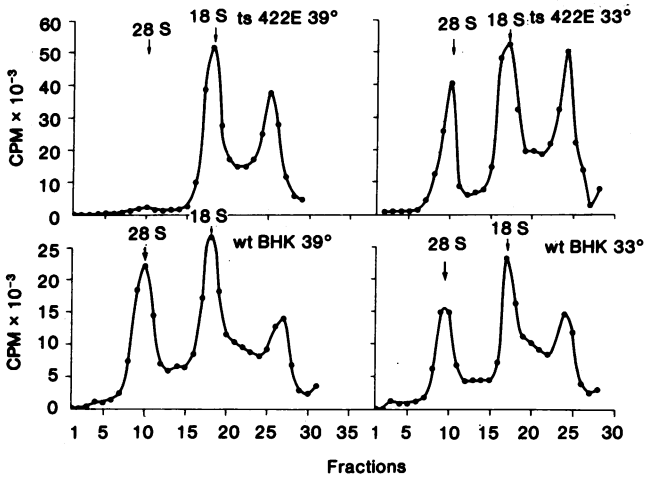

FIG. 2. Production of cytoplasmic rRNA in ts 422E and wt BKH cells at 33° and 39°. Cells were plated at 33° and 39°. After 2 days they were labeled for 2 hr at 33° and 1.5 hr at 39° with [³H]uridine (1 μCi, 0.2 μg/ml). At the end of the labeling period the cells were washed twice with cold, isotonic Tris-buffered saline, then harvested and fractionated as described in *Methods*. The cytoplasm from 1 × 10⁷ cells was analyzed on SDS–sucrose gradients. Sedimentation is from right to left. The *arrows* indicate the position in the gradients of the *A* 260 nm peaks of 28S and 18S rRNA, obtained from collection of the gradients through an automatically recording Gilford spectrophotometer.

A more detailed analysis of the situation seen in the cytoplasm of ts 422E cells at 39° is given in Fig. 3. In these experiments, cells were incubated at 39° for 2 days and pulsed with [³H]uridine for 30 min. The label was chased for various amounts of time in the presence of an excess of unlabeled uridine. The cytoplasmic fraction was analyzed as in Fig. 2. As a consequence of the processing of rRNA in the nucleoli, the 18S rRNA is rapidly transferred to the cytoplasm, while the 28S rRNA is found in the cytoplasm only later. For this reason, some time is required before radioactively labeled rRNA reaches in the cytoplasm the steady-state ratio 28S RNA/18S RNA of about 2:1 (4). Fig. 3 shows that in ts 422E cells, synthesis and appearance of 18S RNA in the cytoplasm in essentially identical to that of wild-type cells. When we measure the ratio of radioactivity in 28S rRNA to that in 18S rRNA, we see that while the ratio 28S/18S in wild-type cells reaches a steady-state value of about 2 within 4–5 hr, appearance of 28S RNA in the cytoplasm of ts 422E is greatly delayed, and the ratio cpm 28S RNA/cpm 18S RNA never reaches a 2:1 value, even with a 24-hr chase. From the rate of appearance of 28S RNA in the cytoplasm during the first 4 hr, the inhibition of 28S rRNA formation in ts 422E cells at 39° was calculated to be about 95%.

From these experiments it is clear that at high temperature ts 422E has a defect in ribosomal RNA formation that prevents the appearance of 28S RNA in the cytoplasm. As judged by the absorbance at 260 nm, the amounts of 28S and and 18S RNA in the cytoplasm, which are about equimolar at 33°, reach a 28S/18S molar ratio of about 1:4 in ts 422E cells 2–3 days after shift to 39°.

Production of Ribosomal Subunits. As expected, the production of 40S and 60S ribosomal subunits has the same pattern shown for 18S and 28S RNA: the production of the 40S ribosomal subunit is normal at both temperatures, while that of the 60S subunit is normal at 33° and greatly impaired at 39°.

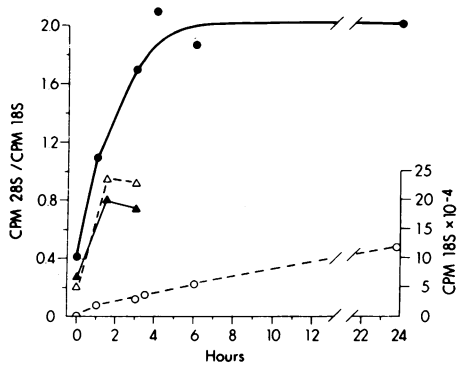

FIG. 3. Appearance of rRNA in the cytoplasm of ts 422E cells at 39°. ts 422E and wt BHK cells growing at 33° were shifted to 39°. Two days later they were labeled with [³H]uridine (3 μCi, 0.08 μg/ml) for 30 min. At the end of the labeling period (0 time), the cells were washed and incubated with unlabeled uridine (50 μg/ml). At the times indicated the cells were harvested and the cytoplasmic fraction was prepared and analyzed on SDS–sucrose gradients as in Fig. 2. The right ordinate indicates the radioactivity appearing in the 18S RNA region of the gradient, expressed as cpm/1.5 × 10⁷ cells. ▲——▲, wt BHK; Δ- - -Δ, ts 422 E. The left ordinate gives the ratio of radioactivity in the 28S to the 18S region. ●——●, wtBHK; O- - -O, ts 422E. The figure is a compendium of data from three different experiments.

Temperature Shifts. To determine how soon after shift to 39° the inhibition of the production of 28S rRNA was established, we performed the following experiment: the cells were shifted to 39°; at different times after shift they were labeled for 1.5 hr with [³H]uridine, and the cytoplasmic fraction was analyzed on SDS–sucrose gradients.

As early as 2–3 hr after shift (Fig. 4) the amount of newly synthesized RNA appearing in the cytoplasm is already re-

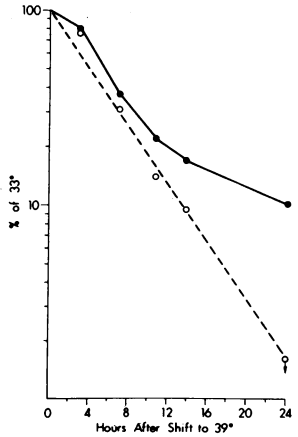

FIG. 4. Production of 28S rRNA in ts 422E cells after shift to 39°. Cells growing at 33° were shifted to 39°. 45 Min before the times indicated they were labeled for 1.5 hr with [³H]uridine (1 μCi, 0.2 μg/ml). One sample was labeled at 33° for 2 hr. At the end of each labeling period cells were harvested and fractionated. The cytoplasmic fraction was analyzed on SDS–sucrose gradients. ●——●, ratio: cpm 28S RNA/cpm 18S RNA, expressed as % of the 33° ratio. O- - -O. This curve was obtained by subtraction from each experimental point the ratio of the last point (24 hr after shift). Data are expressed as % of the 33° value.

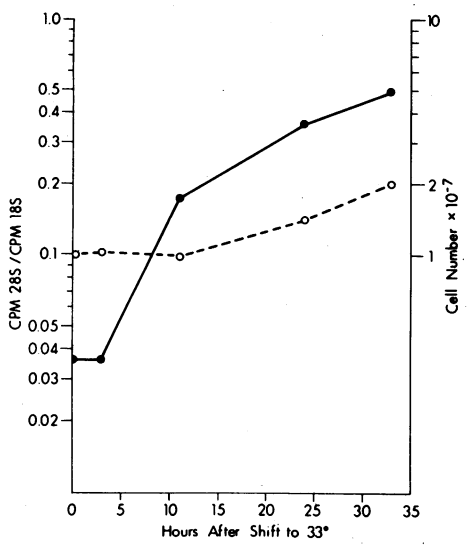

FIG. 5. Resumption of production of 28S RNA in ts 422E cells after shift from 39° to 33°. After 26 hr at 39°, cells were shifted to 33°. 1 hr before the times indicated the cells were labeled for 2 hr with [^{3}H]uridine (1 μCi, 0.2 μg/ml). One sample was labeled at 39° for 1.5 hr. At the end of the labeling periods the cells were harvested and counted, and the cytoplasm was prepared and analyzed on SDS-sucrose gradients. ●——●, ratio: radioactivity in 28S RNA/radioactivity in 18S RNA. O- - -O, number of cells obtained from eight 100-mm petri dishes at each point.

duced to 80% of the amount produced at 33°. It then continues to decrease until it levels off at about 16 hr after shift-up. If we assume that the residual production of 28S RNA after 24 hr at 39° is caused by the leakiness of the mutation, this value can be subtracted from the other points to construct a curve that indicates the inactivation rate of the ability of ts 422E to produce 28S RNA upon shift-up. This curve shows that the impairment of 28S RNA production starts as soon as the cells are shifted to 39°, and the capacity to produce 28S RNA is lost exponentially upon exposure to 39° with a half-life of 4 hr. These experiments indicate that the ts 422E rRNA defect is not a consequence of the inability of the cells to grow at 39°, as they stop growing only 14–18 hr after shift. They also suggest that the ts gene-product of ts 422E cells, once synthesized at the permissive temperature, remains functional also at the nonpermissive temperature, while it does not function when synthesized at 39°.

We also determined the time required for resumption of 28S RNA production and cell division when the cells are shifted back from 39° to 33°. Fig. 5, which gives the results of such an experiment, shows that the time required to reach a normal production of 28S RNA upon shift-down is rather long: only in about 24–28 hr does the appearance of newly synthesized 28S RNA in the cytoplasm approach a normal rate. It is important to notice that a normalization in the production of 28S RNA precedes the resumption of cellular multiplication, suggesting that the defect in rRNA production is responsible for the inability of ts 422E to grow at 39°.

Revertants of ts 422E. We have shown that ts 422E has two ts phenotypic defects, one of growth the other of 28S rRNA production. To test whether the two phenotypic defects could be dissociated, we examined some revertants of ts 422E, either spontaneous or induced with nitrosoguanidine. If temperature sensitivity of growth were produced by the same mutation responsible for the rRNA defect, all the revertants tested, selected on the basis of their ability to multiply at 39°, would show a normal pattern of RNA formation. This was indeed the case.

Mechanism(s) Responsible for Defective Production of 28S rRNA. The reduced appearance of 28S rRNA in the cytoplasm of ts 422E at 39° could be caused either by a defect in rRNA processing, which interferes with the intranucleolar production of 28S RNA (10), or by a defect in the transport of the 28S rRNA into the cytoplasm. In the first case, 28S RNA should not be found in the nucleus, while in the second case it should accumulate in the nucleolus or in the nucleus. These possibilities were tested by fractionating cells into nucleolar, nucleoplasmic, and cytoplasmic fraction and analyzing them by SDS–sucrose gradients or polyacrylamide-gel electrophoresis.

Fig. 6 shows the results of an experiment in which cells were labeled for 20 hr at 39° and the nucleolar and cytoplasmic fraction was analyzed on 2.6% acrylamide gels. The

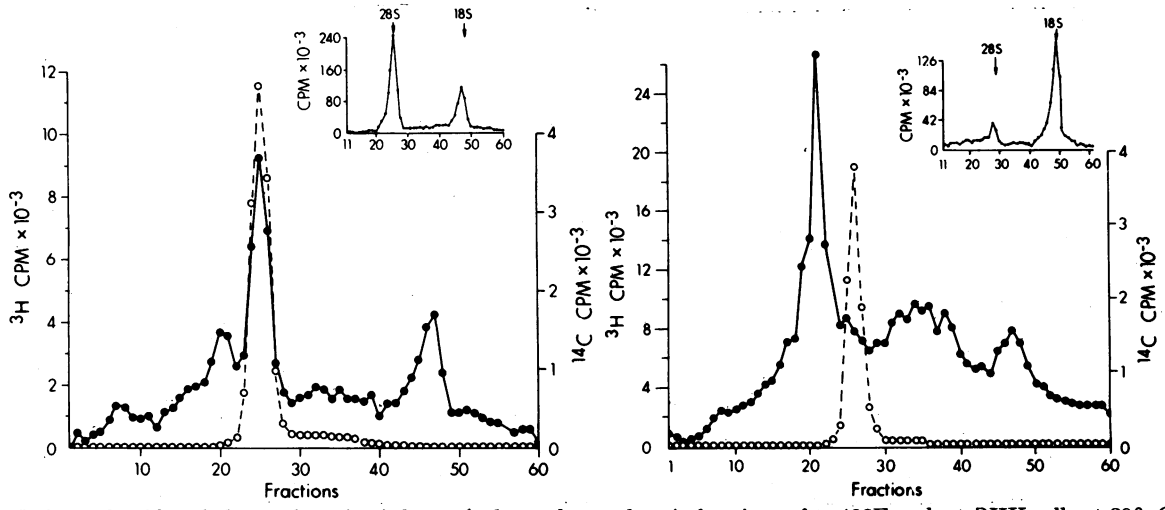

FIG. 6. Polyacrylamide-gel electrophoresis of the nucleolar and cytoplasmic fractions of ts 422E and wt BHK cells at 39°. Cells were shifted to 39°, and after 26 hr they were labeled for 20 hr with [^{3}H]uridine (3 μCi, 5 μg/ml). Cytoplasmic and nucleolar RNA were purified as described in *Methods* and run on 2.6% acrylamide gels for 5 hr at 5 mA/tube, together with a ^{14}C-labeled marker of 28S RNA (O- - -O). *Left:* nucleolar RNA from wt BHK cells. *Right:* nucleolar RNA from ts 422E. The *insets* represent the data for cytoplasmic RNA. All cpms have been normalized to correspond to the nucleolar or cytoplasmic fraction from 6 × 10^6 cells. Electrophoresis is from left to right.

mutant does not have labeled 28S rRNA in the nucleolus, and the radioactivity in the 32S peak is much higher than in wild-type cells. From these and other experiments the amount of 32S RNA accumulating in the nucleoli of ts 422E cells was calculated to be about 10 times that of wild-type cells. However, this accumulation did not quantitatively account for the amount of labeled 28S rRNA missing from the cytoplasm (see *inset* in Fig. 6). Some 32S RNA was probably degraded (4).

From different experiments, we can conclude, as expected from the normal production of 18S rRNA, that the synthesis of the large rRNA precursor molecules in the nucleolus is normal in ts 422E cells, both at 33° and 39°. Essentially no 28S rRNA was ever found in the nucleolus or the nucleoplasm of ts 422E cells at 39°, and we could rule out that 28S rRNA was synthesized and broken down either in the nucleus or in the cytoplasm for short labeling periods gave undetectable amounts of radioactivity in the 28S RNA region. Furthermore, after chase of radioactivity, the small amount of 28S RNA appearing in the cytoplasm was perfectly stable. 32S RNA accumulated to some extent into the nucleolar fraction of ts 422E cells at 39°.

It can be concluded that ts 422E does not produce 28S rRNA, and that the rRNA processing is blocked at the 32S RNA level.

DISCUSSION

Our results show that ts 422E cells have a specific defect in rRNA processing at 39°: 32S RNA is not converted to 28S RNA and 60S ribosomal subunits are not produced. rRNA transcription and production of 18S RNA and 40S ribosomal subunits are not affected. This defect in processing leads to an abnormal ratio of large to small ribosomal subunits in the cytoplasm of ts 422E cells at 39°, but does not appear to affect other macromolecular syntheses, at least within 2–3 days after shift-up.

From our experiments, it appears quite likely that ts 422E cells have a single mutation responsible for both ts rRNA processing and ts growth. We can exclude that the block in 32S RNA processing is a consequence of the fact that the cells stop dividing at 39°. Evidence for this conclusion is provided by the temperature-shift experiments, and by the fact that other ts BHK mutants do not show any impairment of rRNA production at 39° (unpublished results). We can also exclude that ts 422E cells have two ts mutations in different genes, as restoration of growth at 39° in 422E revertants was always associated with normal production of mature rRNA species.

It is, however, possible that the block in 32S RNA processing could be a consequence of a mutation in a different area of the cell metabolism. This hypothesis, however, appears unlikely in view of the short time required for inhibition of the 28S RNA production in the mutant, after shift to high temperature. Moreover, we did not find any impairment of other steps necessary to the synthesis of the mature rRNA species, while it is known that when rRNA processing is blocked at some level by various agents (base analogues or inhibitors of RNA and protein synthesis), there is always a general impairment of the rRNA synthesis as well as of the overall processing (4, 11, 12), and the same behavior has also been shown for several ts mutants of yeast (13). It is therefore reasonable to think that only a specific mutation, directly affecting one step of the process, could give such a result.

It is possible to make some speculation on what mutation could be responsible for the ts phenotype of 422E cells. On the basis of complementation experiments (1), the mutation is recessive, and the temperature-shift experiments are in agreement with the alteration of a protein that is functional when synthesized at the permissive temperature, but does not assume a functional configuration when synthesized at 39°. At present, the most likely possibilities are: (*a*) a ts processing enzyme; (*b*) a ts ribosomal protein that could also be involved in processing; (*c*) a ts defect of methylation.

The behavior of ts 422E is also interesting in view of its possible relevance for the elucidation of the cellular mechanisms that regulate macromolecular synthesis and cell division. ts 422E cells stop multiplying soon after shift to the high temperature, as the cells perform on average only one division at 39°, while the overall rate of protein synthesis is not affected, and also 4-5S RNA and heterogenous nuclear RNA are synthesized at a normal rate. It is therefore possible to imagine that the mutant stops growing because of a more specific mechanism that correlates ribosome production to the maintenance of normal cell growth. While this and other points have to be investigated, it is worth mentioning that, aside from the potential importance of these cells for the study of rRNA biosynthesis, the fact that conditional lethal mutants of mammalian cells can be characterized gives substance to the idea that ts mutations can be used to study the genetics and biochemistry of somatic animal cells.

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