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Genetics

Temperature-sensitive RNA polymerase II mutations in Chinese hamster ovary cells

(α-amanitin resistance/nitrosoguanidine mutagenesis/cell hybridization/reversion/RNA nucleotidyltransferase)

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ABSTRACT **Mutant Chinese hamster ovary cell lines temperature-sensitive (TS) for growth and containing TS mutations in RNA polymerase II (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) have been isolated. Wild-type cells were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and a population of cells possessing mutations in RNA polymerase II was initially selected by isolating α-amanitin-resistant clones at 34°. Of 168 such α-amanitin-resistant isolates screened for temperature sensitivity, nine were TS for growth at 39.5°. By examining the behavior of the α-amanitin resistance of these TS cell lines in somatic cell hybrids, the TS mutation in a number of them was shown to be in RNA polymerase II. Hybrid cells obtained by the fusion of the TS and α-amanitin-resistant cells with cells possessing α-amanitin-sensitive polymerase II grew at both 34° and 39.5°; the TS mutations were recessive. At 34° all the hybrids were α-amanitin-resistant and possessed a mixture of α-amanitin-resistant and sensitive polymerase II. At 39.5° the α-amanitin-resistant polymerase II activities in hybrids of four of the TS cell lines were lost; these four lines were α-amanitin-sensitive and possessed only α-amanitin-sensitive polymerase II. Temperature-insensitive revertants of two of these mutants were isolated. Reversion of the TS phenotype for mutants TsAma^R-1 and TsAma^R-8 was accompanied by an alteration in the level of α-amanitin resistance of the RNA polymerase II activities in the revertant cells. Together these data provide convincing evidence that TS mutations in RNA polymerase II can be coselected with α-amanitin resistance.**

Investigation of the properties of the multiple forms of DNA-dependent RNA polymerases (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) present in animal cells can be facilitated by the availability of mutant cell lines containing altered RNA polymerase activities. The cytotoxicity of α-amanitin has provided the means of isolating mutant cells that contain altered, α-amanitin-resistant, RNA polymerase II activities. Such mutants have been obtained in a number of continuous cell lines, including Chinese hamster ovary (CHO) (1, 2), rat myoblast L6 (3, 4), baby hamster kidney (BHK) (5), and mouse myeloma (6), and in short-term cultures of human diploid fibroblast explants (7). Characterization of these and other variant cell lines (for review see ref. 8), together with the more recent studies of Milman *et al.* (9) and Steinberg *et al.* (10) demonstrating electrophoretically altered proteins in variant cell lines, has provided convincing evidence that such mammalian cell variants in many cases are indeed the result of classical Mendelian mutational events. The availability of the α-amanitin-resistant RNA polymerase II mutants has, in addition, allowed a study of the regulation of intracellular polymerase II activity in both CHO (11) and L6 (12, 13) cell lines. However, it has become clear that a better understanding of the mechanisms regulating both the activity and transcriptional selectivity of RNA polymerases will require the isolation of additional mutations affecting transcription in mammalian cells. In particular, the isolation of conditional-lethal temperature-sensitive (TS) mutations in RNA polymerases would be desirable.

The isolation of mutations affecting specific gene functions in bacteria has been facilitated by development of techniques for localized mutagenesis (14). In general these techniques are not readily applicable for the isolation of specific mutations in mammalian cells. However, the comutagenic selection procedure described by Oeschger and Berlyn (15) could potentially offer a method to obtain populations of mammalian cells enriched for mutations in specific gene loci. The present studies therefore were undertaken in part to test the feasibility of a coselection approach using nitrosoguanidine as the mutagen and α-amanitin as the selection agent for mutations in the RNA polymerase II locus. It was expected that amongst a wide spectrum of RNA polymerase II mutants might be some with both α-amanitin-resistant and TS phenotypes. I show here that nitrosoguanidine does induce α-amanitin resistance mutations in CHO cells and that a number of such α-amanitin-resistant isolates are also TS for growth. By analyses of several of these TS mutants, using somatic cell hybridization and the characterization of revertants of the TS phenotype, I have clearly established that TS mutations can be coselected with α-amanitin resistance and that some of these TS mutations are mutations in the enzyme RNA polymerase II.

MATERIALS AND METHODS

Cells and Cell Culture. The CHO cell line used as parent in the selection of α-amanitin-resistant and temperature-sensitive mutants was the wild-type CHO cell line (16) auxotrophic for proline (Pro$^-$). A CHO cell line auxotrophic for glycine, adenosine, and thymidine (Pro$^+$ GAT$^-$) (17) was used as the complementary α-amanitin-sensitive parent in cell hybridization experiments.

Cells were grown either in suspension culture or as monolayer cultures at 34° in α medium (18) containing 10% fetal calf serum and were cloned in 96-well Linbro tissue culture dishes as previously described (2).

Mutant Selections. CHO cells in logarithmic-phase growth in suspension culture at 34° were treated for 3 hr with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich) at a concentration of 0.1 μg/ml and then plated (10^6 cells per 100-mm dish) in 10 ml of medium at 34°. After 3 days

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Abbreviations: CHO, Chinese hamster ovary; TS, temperature-sensitive; Ama^R, α-amanitin-resistant; Ama^S, α-amanitin-sensitive; Pro$^-$, auxotrophic for proline; GAT$^-$, auxotrophic for glycine, adenosine, and thymidine.

the medium was replaced with 10 ml of medium containing α-amanitin (Mallinckrodt, Canada, Ltd.) at 1.0 μg/ml, and this medium was changed once after a further 7 days. Surviving colonies were picked after 12–16 days, grown in the absence of α-amanitin for 2–4 days at 34°, and then tested for the presence of TS phenotypes by plating 10^3 cells of each isolate at 34° and 39.5°. TS isolates were, in turn, grown up and cloned at 34°, and subclones were retested for their α-amanitin-resistant (Ama^R) and TS phenotypes. The Ama^R and TS mutants have been designated as $TsAma^R$-1, $TsAma^R$-8, etc., according to their isolate number in the α-amanitin resistance selection.

Revertants of the TS cell lines $TsAma^R$-1 and $TsAma^R$-8 were obtained following similar mutagenesis with nitrosoguanidine, growth for 3 days at 34°, and then selection in monolayer cultures at 39.5°.

Cell Hybridization. Hybrids between the Ama^R TS (Pro^-) isolates and Ama^S (GAT^- Pro^+) cells were constructed by addition of 44%, (wt/wt) polyethyleneglycol 6000 (BDH)/10% (vol/vol) dimethylsulfoxide (19) to monolayer cultures in 35-mm dishes consisting of 10^6 cells of each parent, and by selection at 34° in α medium lacking proline, glycine, adenosine, and thymidine. Once isolated, hybrid colonies were cloned and karyotyped to verify that they were indeed pseudotetraploid. Mock crosses of each parental line to itself indicated that the frequency of revertants ($<10^{-5}$) in each population was less than the hybridization frequency ($\sim 3 \times 10^{-3}$).

RNA Polymerase Characterization. RNA polymerase activities present in the various cell lines were resolved by DEAE-Sephadex chromatography and assayed as previously described (2).

RESULTS

Selection of α-amanitin-resistant, temperature-sensitive CHO cells

The procedure for localized mutagenesis, termed "comutagenic selection" by Oeschger and Berlyn (15), involves nitrosoguanidine mutagenesis and positive selection for a specific phenotype. Nitrosoguanidine is thought to cause multiple mutations, preferentially in the region of the selection marker. I have attempted to use this approach to coselect TS mutations in RNA polymerase II. Wild-type CHO cells were mutagenized with nitrosoguanidine and α-amanitin-resistant isolates were selected at the permissive temperature of 34°. Each of these α-amanitin-resistant isolates was then screened for conditional-lethal TS mutations by testing its ability to grow at 39.5°.

Nitrosoguanidine is a potent mutagen for the RNA polymerase II locus in CHO cells. With mutagenesis treatments that reduced the relative plating efficiency of the cells to 35% and 16% in two different selections, the frequency of recovery of colonies resistant to α-amanitin at 1.0 μg/ml was 4×10^{-6}, which was approximately 25-fold greater than the frequency for unmutagenized populations. The first trial selection (2.5×10^6 surviving cells plated after mutagenesis) yielded 8 α-amanitin-resistant colonies, of which one, designated $TsAma^R$-8, was TS for growth. In the second, much larger, selection (4×10^7 surviving cells plated after mutagenesis), of 160 α-amanitin-resistant isolates, a total of 8 (5%) were TS for growth. Individual subclones of all but one of these nine original isolates (each of them picked from different plates and hence most probably independent) retained both their α-amanitin-resistant and temperature-sensitive phenotypes after growth at 34° in the absence of α-amanitin for 30–60 days and after subsequent recloning.

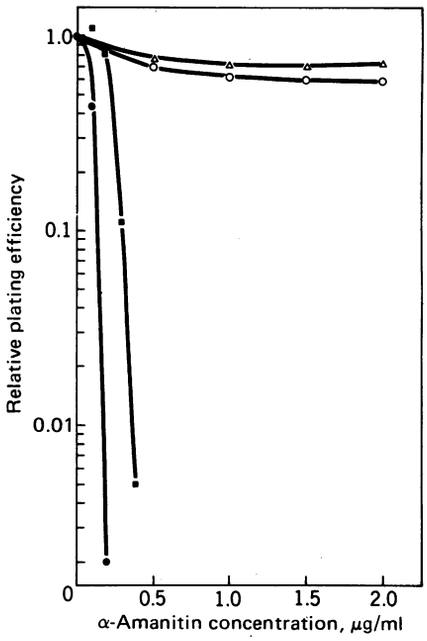

FIG. 1. Sensitivity of growth of various CHO cell lines to inhibition by α-amanitin. The relative plating efficiencies of wild-type CHO (■), the TS mutant $TsAma^R$-8 (△), and the $TsAma^R$-8 × GAT^- Pro^+ hybrid cells (○), all at 34°, and the $TsAma^R$-8 × GAT^- Pro^+ hybrid cells at 39.5° (●) were determined by plating each cell line (10^3 cells/60-mm dish) in 5 ml of medium containing increasing concentrations of α-amanitin. Colonies were fixed, stained, and counted after 10–12 days growth.

Behavior of the TS mutations in hybrid cells

The temperature-sensitive mutations in the α-amanitin-resistant cell lines could be TS mutations either in RNA polymerase II or in any other unrelated cellular function. These possibilities can be distinguished by examining the behavior of the α-amanitin resistance of the Ama^R TS mutants in hybrid cell lines at 34° and 39.5°. α-Amanitin resistance behaves codominantly (20); that is, both wild-type and mutant genes are expressed in hybrid cells. Ama^R/Ama^S hybrid cells are α-amanitin-resistant and contain a mixture of Ama^R and Ama^S forms of RNA polymerase II (2, 11, 20). Thus, in a hybrid cell containing a parental genome such as that of $TsAma^R$-8, if the TS mutation in $TsAma^R$-8 is in RNA polymerase II, the $TsAma^R$-8 × Ama^S hybrid cells should not grow at 39.5° in the presence of α-amanitin. The wild-type Ama^S polymerase II will be inactivated by α-amanitin and the mutant Ama^R allele will not be expressed at this temperature. At 34° the hybrid cells should grow in α-amanitin because the mutant Ama^R allele will be expressed. On the other hand, if the TS mutation involves another gene and is recessive, then the hybrid cells will grow at both 34° and 39.5° in the presence of α-amanitin, because the TS gene will be suppressed and the Ama^R gene will act dominantly as usual.

I therefore constructed the appropriate hybrids as described in *Materials and Methods* and studied their properties as described below in detail for the mutant $TsAma^R$-8. In Fig. 1, as expected, both the mutant $TsAma^R$-8 and the $TsAma^R$-8 × Ama^S hybrid cells can be seen to be α-amanitin-resistant at 34°, plating at >60% efficiency in α-amanitin at 2 μg/ml. Under the same conditions, parental wild-type CHO cells were effectively killed with α-amanitin concentrations as low as 0.2 μg/ml. The TS mutation in $TsAma^R$-8 × Ama^S hybrid cells behaved recessively, because the cultures grew equally well at 34° and 39.5°. However, as seen in Fig. 1, the hybrids had a markedly increased sensitivity to α-amanitin when grown at

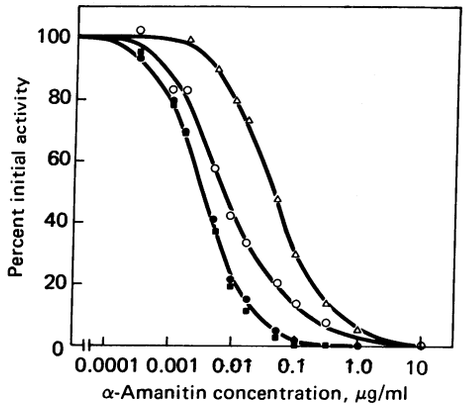

FIG. 2. Inhibition of RNA polymerase II activities by α-amanitin. DEAE-Sephadex-purified RNA polymerase II peak fractions from wild-type CHO cells (■), TsAma^R-8 cells (△), and TsAma^R-8 × GAT$^-$ Pro$^+$ hybrid cells (○), each grown at 34°, and TsAma^R-8 × GAT$^-$ Pro$^+$ cells grown at 39.5° (●) for 3 days were assayed for 15 min at 30° in the presence of increasing concentrations of α-amanitin.

39.5°. The hybrid cells, α-amanitin-resistant at 34°, were killed at 39.5° by concentrations of α-amanitin similar to those that were cytotoxic for the wild-type CHO cells containing only Ama^S polymerase II. Thus, at 39.5° the α-amanitin-resistant phenotype of the hybrids was lost, indicating that the TS mutation in TsAma^R-8 is in RNA polymerase II.

In order to provide more stringent evidence on the nature of RNA polymerases in the mutant and hybrid cells, I next examined the polymerase II activities in these cell lines. As shown in Fig. 2, the mutant TsAma^R-8 does contain an altered, more α-amanitin-resistant RNA polymerase II activity. Extracts of both the parental Ama^S and TsAma^R-8 cells grown in the absence of α-amanitin were chromatographed on DEAE-Sephadex. The partially purified polymerase II peak fractions were then examined for sensitivity to α-amanitin. The polymerase II activity from the mutant TsAma^R-8 required concentrations of α-amanitin 15- to 20-fold higher than the concentration required to inhibit wild-type Ama^S polymerase II. As expected, titration of the polymerase II from TsAma^R-8 × Ama^S hybrid cells grown at 34° in the absence of α-amanitin was intermediate between that for TsAma^R-8 and wild-type polymerase II. These hybrid cells contained approximately equal amounts of the resistant polymerase II contributed by the parental TsAma^R-8 cells and the α-amanitin-sensitive polymerase II contributed by the Ama^S cells. However, as shown in Fig. 2, when these same hybrids were grown at 39.5°, the RNA polymerase II activity had a sensitivity to α-amanitin exactly like that of wild-type cells. Growth of the hybrid cells at the nonpermissive (for TsAma^R-8) temperature of 39.5° had eliminated the α-amanitin-resistant component of RNA polymerase II. These experiments demonstrate the temperature-sensitive nature of the α-amanitin-resistant polymerase II in TsAma^R-8 cells.

The TS defect in a number of the other α-amanitin-resistant TS isolates was examined in a similar manner in hybrid cells. As indicated in Table 1, growth of the hybrid cells formed by fusion of the mutant clones TsAma^R-1, TsAma^R-8, TsAma^R-38, and TsAma^R-67 with Ama^S cells was in each case α-amanitin-resistant at 34° but extremely α-amanitin-sensitive at 39.5°. Furthermore, in these hybrids grown at 39.5° the RNA polymerase II activity had in each case lost its α-amanitin-resistant component (data not shown). Thus each of these isolates behaved like TS RNA polymerase II mutants. In contrast, three other TS mutants appeared to contain unlinked TS mutations.

Table 1. Effects of temperature on growth of hybrid cell lines in α-amanitin

Mutant cell line hybridized	Relative plating efficiency of hybrid cells in α-amanitin, %	
	34°	39.5°
TsAma^R-1	38	<0.5
TsAma^R-8	62	<0.2
TsAma^R-38	88	<0.2
TsAma^R-67	82	<0.1
TsAma^R-76	14	17
TsAma^R-78	20	45
TsAma^R-94	10	20

Hybrid cells (10^3/60-mm dish), formed by fusing each of the indicated mutant cell lines with α-amanitin-sensitive (GAT$^-$ Pro$^+$) CHO cells, were plated at either 34° or 39.5° in the absence of α-amanitin or in α-amanitin at 1.0 μg/ml.

Hybrids of the mutants TsAma^R-76, TsAma^R-78, and TsAma^R-94 remained α-amanitin-resistant at both 34° and 39.5°.

TS$^+$ revertant cell lines

In order to further demonstrate the close linkage of some of these TS and α-amanitin resistance mutations, the effect of reversion of the TS phenotype on the α-amanitin resistance was examined. For two of the TS cell lines, TsAma^R-1 and TsAma^R-8, TS$^+$ revertant clones able to grow at 39.5° were selected after nitrosoguanidine mutagenesis of the TS parents. The frequency of reversion was in the order of 10^{-5} to 10^{-6}. Several representative clones from each line were picked and tested for their RNA polymerase II properties. The revertant of TsAma^R-1 did not grow in concentrations of α-amanitin greater than 0.1 μg/ml and its RNA polymerase II had a sensitivity to inhibition by α-amanitin exactly like the polymerase II of wild-type CHO cells (Fig. 3*A*). The revertant of TsAma^R-8 similarly showed an altered α-amanitin sensitivity. In this case, however, growth of the TS$^+$ revertant cell line remained α-amanitin-resistant. Its relative plating efficiency in α-amanitin was similar to that of the parental TsAma^R-8 cell line. However, inhibition of this revertant's RNA polymerase II activity required concentrations of α-amanitin less than those needed to inhibit the parental TsAma^R-8 polymerase II, but greater than those needed to inhibit wild-type polymerase II (Fig. 3*B*). Thus, reversion of the TS phenotype was in both cases accompanied by an alteration in the level of α-amanitin resistance of the RNA polymerase II activities in the TS$^+$ revertant cells. These results provide convincing evidence that the mutations altering the TS phenotype of these two cell lines involved changes in the enzyme RNA polymerase II.

DISCUSSION

Mutant CHO cells containing TS mutations in RNA polymerase II have been obtained after nitrosoguanidine mutagenesis and positive selection of RNA polymerase II mutations. A number of α-amanitin-resistant isolates selected at 34° were unable to grow at 39.5°. The TS defect in four of seven such TS mutants examined was shown to reside in the enzyme RNA polymerase II. For the initial characterization of the TS defects I relied upon the behavior of these mutations in somatic cell hybrids. Fusion of the mutants TsAma^R-1, TsAma^R-8, TsAma^R-38, and TsAma^R-67 with cells containing wild-type RNA polymerase II resulted in Ama^R/Ama^S hybrid cells that lost their codominantly expressed α-amanitin resistance at the nonpermissive

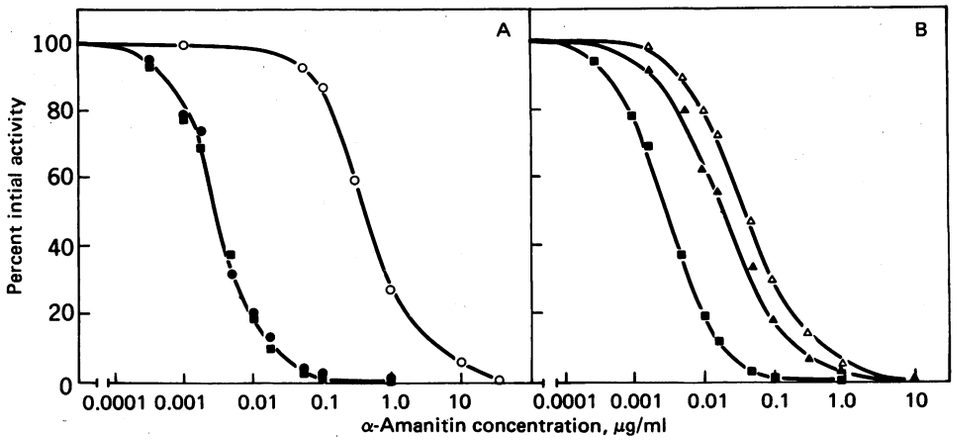

FIG. 3. Inhibition of TS mutant and TS$^+$ revertant cell RNA polymerase II activities by α-amanitin. DEAE-Sephadex-purified polymerase II activities of each cell line grown at 34° were assayed as described in Fig. 2. (*A*) ■, Wild-type CHO cells; ○, TsAma^R-1; ●, TS$^+$ revertant of TsAma^R-1. (*B*) ■, Wild-type CHO cells; △, TsAma^R-8; ▲, TS$^+$ revertant of TsAma^R-8.

temperature of 39.5°. The α-amanitin resistance of the hybrid cells was shown to be TS by growth experiments (Fig. 1 and Table 1) and by measurement of their RNA polymerase II activities (Fig. 2). The behavior of these Ama^R and TS mutations in the cell hybridization studies suggests that in the four TS cell lines the Ama^R and TS mutations reside in the same complementation group, most likely the α-amanitin-binding, 140,000-dalton subunit (21) of polymerase II. These four TS mutants are not, however, independent isolates of the same TS mutant, because the level of α-amanitin resistance of polymerase II varied among the mutants (for example compare the activities of RNA polymerase II in the mutants TsAma^R-1 and TsAma^R-8 in Fig. 3*A* and *B*).

The isolation and characterization of TS$^+$ revertants of two of these mutants provides further evidence that the TS defects affecting growth of these mutant cell lines are in RNA polymerase II. Reversion of the TS phenotype in both instances was accompanied by an alteration in the α-amanitin sensitivity of polymerase II. Because the TS$^+$ reversion event restored wild-type α-amanitin sensitivity to the polymerase II of revertants of TsAma^R-1, most likely a single point mutation is responsible for both the increased α-amanitin resistance and TS phenotype of the TsAma^R-1 cells. For TsAma^R-8, either a single mutation or two closely linked mutations may have led to its drug-resistant and TS phenotype; the polymerase II in this revertant also had an altered drug sensitivity, albeit different from that of either wild-type or TsAma^R-8 polymerase II.

Nitrosoguanidine induces double mutations at a high frequency in prokaryotes (22), and these mutations are often closely linked on the bacterial chromosome. In fact, nitrosoguanidine mutagenesis coupled with positive selection for mutations at a specific locus is the basis of a technique of localized mutagenesis described by Oeschger and Berlyn (15). Although the present studies were undertaken in part to test the feasibility of such a nitrosoguanidine comutagenic selection in mammalian cells, they have not provided compelling evidence for a clustering of multiple mutations at a single genetic region. Both the α-amanitin-resistant and TS phenotypes of RNA polymerase II may be the result of a single mutation in each of these TS cell lines. Studies of Dawes *et al.* (23) have indicated that most of the coselected TS mutations induced by nitrosoguanidine in *Saccharomyces cerevisiae* do not map close to the selected marker. Similarly, the mutants TsAma^R-76, TsAma^R-78, and TsAma^R-94 selected in this study (Table 1), while having altered α-amanitin-resistant polymerase II activities, must contain TS mutations in other cellular functions. Nevertheless, in many of the isolates the TS defect is closely linked with α-amanitin resistance, suggesting that the coselection is extremely stringent. A mutation must not only occur in a protein-coding region of DNA to engender a TS phenotype, but, being recessive, must occur in a region of DNA functionally hemizygous. Our previous studies have shown that these CHO cells, although near-diploid in chromosome karyotype analyses (24), have but one functional allele coding for the α-amanitin-binding subunit of RNA polymerase II (2). This functional hemizygosity likely facilitated the isolation of recessive TS RNA polymerase II mutations in CHO cells. It is important to note, however, that similar mutants could be readily isolated in other cell lines such as the rat myoblast L6 (3) or mouse myeloma cell lines (6), because any complementing wild-type RNA polymerase II present in cell lines functionally diploid at this locus can be inactivated by growth of the cells in α-amanitin containing medium. The availability of temperature-sensitive mutations affecting RNA polymerase II activity could aid considerably in studies of the functional role of this enzyme in such a diversity of cell types.

The exact nature of the TS defect in each of these mutant CHO cells remains to be elucidated. Preliminary studies of the mutant TsAma^R-8 suggest that RNA synthesis does not stop abruptly upon shift of the cells to the nonpermissive temperature. Rather, synthesis or assembly of this enzyme may be affected. A range of TS phenotypes affecting RNA synthesis itself as well as enzyme synthesis or assembly has already been noted in studies of TS mutations of *Escherichia coli* RNA polymerase (25–27). The coselection approach employed in these studies, using α-amanitin to enrich for RNA polymerase II mutants in a population of mutagen-treated cells, may yield a similar range of mutant RNA polymerase II phenotypes. Such mutants should prove useful in studies of regulated RNA synthesis in mammalian cells.

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