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Dominant and Semidominant Mutations Leading to Thermosensitivity of Ribonucleic Acid Biosynthesis in *Saccharomyces cerevisiae*

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Different dominant thermosensitive mutations affecting the same gene were selected in *Saccharomyces cerevisiae*. Ribonucleic acid (RNA) synthesis decreased rapidly and markedly at 37 C in all the mutants whether they were in a homozygous or a heterozygous state. Protein biosynthesis was at first unaffected and then decreased slowly, stopping after 5 h. Measurements of RNA biosynthesis in isolated nuclei as well as in vitro activities of RNA polymerases A and B at 22 and 37 C failed to reveal any difference between mutants and the wild type. Analysis of the nature of the residual RNAs synthesized at the high temperature in the mutants showed a small relative increase in the messenger RNA fraction, but it was not sufficient to indicate a specific inactivation of RNA polymerase A activity. The results suggest an impairment in a common regulatory element for all RNA polymerases acting at the level of the initiation of transcription. Similar mutants with a semidominant phenotype were obtained in which the lesions were in two other unlinked loci.

The existence of multiple forms of ribonucleic acid (RNA) polymerases in yeast as in other eukaryotes (1) raises an interesting problem concerning their specificities and the general regulation of RNA biosynthesis. Physiological and biochemical studies have revealed a specific role for RNA polymerase A in ribosomal RNA biosynthesis (15), for RNA polymerase B in messenger RNA biosynthesis (22), and more recently for RNA polymerase C in transfer RNA biosynthesis (20). Isolation of different thermosensitive mutants impaired for each of the three activities would be clearly of great help in confirming these assignments and in uncovering the cross-regulatory links between the synthesis of the different RNA species. For this reason we have tried, as have many other laboratories, to select such mutants. This paper presents the different approaches used and some details about a class of dominant mutations found during the selections. Although other explanations cannot be definitely excluded, this class of mutants does not seem to be impaired in any specific RNA polymerase activity but in a common step acting at the level of the initiation of transcription.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study were all derived from two isogenic strains FL 100 (a) and FL 200 (α) (11).

Media and culture methods. Yeast nitrogen base (Difco), 6.7 g/liter, supplemented with 2% glucose was the basic minimal medium. For genetic analysis a complete medium with yeast extract (1%), peptone (1%) (Difco), and glucose (2%) was routinely used.

Genetic analysis. The methods described by Mortimer and Hawthorne (14) were used. The mutagenesis by ultraviolet light was always done by a direct irradiation of the plates after spreading with cells from a young stationary culture stored for 1 week in a refrigerator. The time of irradiation was chosen to yield about 1% survival.

Chemicals. Lomofungin was a gift of G. B. Whitfield of the Upjohn Co. and thiolutin was a gift from Pfizer Inc. Uniformly labeled [^{14}C]uracil and [^{14}C]leucine were purchased from the French C.E.A.

Measurement of RNA synthesis in isolated nuclei. A slight modification of the method used by Wintersberger et al. (20) was used. Cells growing exponentially at 22 C in liquid yeast nitrogen base medium were harvested and transformed into protoplasts by snail enzyme in 1 M sorbitol without buffer (8). After centrifugation and washing with 1 M sorbitol, the spheroplasts were suspended in 1 M sorbitol-yeast nitrogen base medium for 1 h at 22 C to allow physiological recovery. The spheroplasts were then centrifuged and suspended in 0.02 M phosphate buffer, pH 6.8, containing 0.5 mM $MgCl_2$ and 18% Ficoll. The unbroken cells were removed by centrifugation for 5 min at 4,000 $\times$ g, and the resultant supernatant fluid was centrifuged at 30,000 $\times$ g for 20 min to remove the nuclei. The nuclei were then resuspended in the 0.02 M phosphate buffer, and RNA biosynthesis was assayed by the method of

Wintersberger et al. (21) as endogenous chromatin activity in the presence of 0.5 M KCl.

Preparation of extracts for RNA polymerase assays. Exponentially growing cells were cooled rapidly, harvested by centrifugation, and washed twice with distilled water and then once with the extraction buffer [0.02 M tris(hydroxymethyl)aminomethane-HCl, pH 8.0, 0.01 M β-mercaptoethanol, 0.01 M $MgCl_2$, 0.001 M ethylenediaminetetraacetic acid, 10% glycerol, 0.3 M ammonium sulfate]. Cells (4 g) were suspended in 4 ml of the above buffer containing 0.004 M phenylmethylsulfonylfluoride and disrupted with an Eaton press. RNA polymerase A was purified up to the *O*-(diethylaminoethyl)-cellulose step by using a microscale adaptation of the purification procedure of Buhler et al. (2). At this stage the enzyme was approximately 50% pure as estimated by sodium dodecyl sulfate-gel electrophoresis (4). RNA polymerase B activity was recovered as a by-product of the above purification and further purified by *O*-(diethylaminoethyl)-cellulose chromatography and glycerol gradient centrifugation (J. M. Buhler, unpublished data). The enzyme was estimated to be 40 to 50% pure.

RNA polymerase assays. RNA polymerase A was assayed by the previously described method (2). RNA polymerase B was assayed as described by Dézélée and Sentenac (4). Unless otherwise indicated, native calf thymus deoxyribonucleic acid purified on nitrocellulose filters (4) was used as template. RNA synthesis was carried out at various temperatures for 20 min and [^{3}H]- or [α-^{32}P]uridine monophosphate incorporation was estimated as previously described (4).

RESULTS

Selection of the mutants. The first approach used was to examine whether mutants resistant to RNA inhibitors were modified in one of the RNA polymerase activities. Very often resistant mutants are only changed in their permeability to the inhibitor or in their ability to destroy it; such unwanted mutations can be numerous enough to prevent the detection of mutants modified in the inhibited enzyme. For this reason we selected mutants that were both resistant to the inhibitor at a low temperature and thermosensitive for growth at a high temperature. Obviously, when these two phenotypes are obtained through a single mutation, this mutation affects an obligatory step in cell physiology that could be expected to be at the RNA polymerase level. Unfortunately, the occurrence of thermosensitive mutations in yeast is high (6, 11) so that very often the resistance to the inhibitor at the low temperature and the thermosensitivity are obtained by two independent mutations. The relatively high frequency of gene conversion during yeast mitosis allows one to discard these double mutants when the strain having the double phenotype is crossed to a wild-type strain of the opposite mating type. Strains resistant to the inhibitor appearing by gene conversion in the diploïds are selected at the low temperature, and these strains are then tested for thermosensitivity. The frequency of gene conversion at two separate loci will be low unless they are extremely closely linked. Therefore, if the double phenotype is due to two different mutations, the converted diploïds will not be thermosensitive, but if it is due to a single mutation, the diploïds will be thermosensitive.

Thiolutin has been described as a strong inhibitor of yeast RNA biosynthesis acting at the level of the RNA polymerases (18). Our wild type is completely inhibited on solid minimal medium by 5 μg of thiolutin per ml. We selected after ultraviolet light mutagenesis strains that were resistant to 7 or 9 μg of thiolutin per ml on solid medium at 22 C. The resistant clones were tested for thermosensitivity by monitoring growth on petri dishes at 22 and 37 C. Of some hundred mutants tested, eleven were thermosensitive; however, only two showed simultaneous segregation of the resistance at the low temperature and of the thermosensitivity in the tetrads obtained after a cross with the wild type. The mutations present in these two strains were shown to be allelic. Measurement of RNA and protein biosynthesis at the restrictive temperature showed a preferential but very late inhibition of RNA biosynthesis (after about 5 h). The molecular mechanism of the mutation has not been studied further owing to the difficulty of interpreting this delayed inhibition of RNA synthesis.

Lomofungin, like thiolutin, has been described as a strong inhibitor of yeast RNA biosynthesis (10). However, when we used it at various concentrations to select resistant strains, we found either complete growth or no growth of the background but never growth of isolated clones. We then saw that lomofungin had a strong killing effect on our wild-type strain, as observed before by Cannon et al. (3); this effect occurred only on exponentially growing cells and not on stationary-phase cells. Moreover, incubation of exponentially growing cells with sodium azide (10^{-2} M) completely prevented the killing. To try to understand whether the killing was correlated with the general activity of the cell or with some specific step, we added either cycloheximide (a protein synthesis inhibitor) or thiolutin (an RNA biosynthesis inhibitor) before the addition of lomofungin. The killing effect was correlated with active RNA synthesis and not with active protein synthesis (Table 1), but more precise exper-

iments are needed to discard completely the possibility that the effect is due to differential entry of inhibitor into the cells.

Since it was impossible to isolate strains able to grow on lomofungin, we tried to find strains that would survive the killing effect of lomofungin at 22 C and which would be thermosensitive at 36 C, with the idea that a change in the primary structure of an RNA polymerase could simultaneously decrease the interaction with lomofungin at 22 C and give thermosensitivity. After a strong ultraviolet light mutagenesis, strain FL 100 was grown for many generations (more than 15) at 22 C to allow selection against any poorly growing strains. Cells in exponential phase were incubated with lomofungin (10 μg/ml) for 30 or 60 min. At this time, the cells were centrifuged, washed once with liquid minimal medium, plated on minimal medium, and incubated at 22 C. After appearance of the colonies, the thermosensitive strains were identified by replica plating and incubation at 36 C. Different mutants were obtained, but study of their RNA biosynthesis failed to reveal any strong inhibition at the restrictive temperature. Another similar selection was undertaken, the only difference being that, after exponential growth at 22 C, the mutated culture was shifted to 36 C for 2 h before lomofungin was added. This was done in the hope that the apparent link observed between RNA biosynthesis and cell killing by lomofungin would lead to a selective survival of thermosensitive mutants impaired in RNA biosynthesis. Eight new thermosensitive strains were thus obtained, one of which showed the interesting properties of dominant thermosensitivity and a strong inhibition of RNA biosynthesis at 36 C. This mutant will be described in more detail later in this paper and has been named RNA 12-1, following the nomenclature used previously by Hartwell et al. for their thermosensitive mutants impaired in RNA biosynthesis (7).

Obtaining this dominant mutant impaired in RNA biosynthesis as well as knowing the dominance of some *Escherichia coli* mutants for RNA polymerase (9) prompted us to reverse the selection applied and to search first for dominant thermosensitive mutants and to examine them afterwards for an impairment in RNA biosynthesis. Unfortunately, when one starts from yeast diploïds to try to find dominant mutations after mutagenesis, one obtains many homozygous recessive mutations obtained through gene conversion. Since the spontaneous high rate of gene conversion is greatly increased by mutagenic agents (16), we decided to mutagenize a haploïd strain auxotrophic for methionine. After the cells had stabilized genetically by growth on methionine-supplemented medium, we crossed them at random with cells from a strain of opposite mating type auxotrophic for leucine and then selected diploïds by their prototrophy on minimal medium at 22 C. The diploïds were grown in exponential phase at 22 C on minimal medium and shifted for 5 h to 37 C, and the growing cells were killed by adding either nystatin (15 μg/ml) (17) or lomofungin (10 μg/ml). After 30 min, the cells were washed, plated on minimal medium, and incubated at 22 C, and the surviving colonies were tested for thermosensitivity by replica plating. Seventy-six thermosensitive diploïds were thus obtained, some of which could have been multiple isolates of the same initial mutant.

Nevertheless, the difference in the phenotypes of thermosensitivity at intermediate temperatures as well as the differences in the rates of conversion or reversion at the high temperature show that they correspond to numerous independent mutations. The frequent appearence of converted wild type at the restrictive temperature is very useful as an indication that one is probably dealing with a dominant and not a double recessive mutant. The amounts of RNA and protein synthesized during 2 h of

TABLE 1. *Differential killing effect of lomofungin*[a]

Survival (%) after incubation with:							
No addition	Lomo	NaN_3	NaN_3 + Lomo at 0 min	NaN_3 + Lomo at 10 min			
100	0.03	100	92	100			
No addition	Lomo	Lomo + Thio at 10 min	Lomo + Cyclo at 10 min	Thio + Lomo at 10 min	Cyclo + Lomo at 10 min	Thio	Cyclo
100	<1	≃1	≃1	96	≃1	86	95

[a] The wild-type strain (FL 100) grown in exponential phase in minimal medium was incubated with the different compounds indicated. NaN_3 was at a final concentration of 10^{-2} M, lomofungin (Lomo) at 10 μg/ml, thiolutin (Thio) at 50 μg/ml, and cycloheximide (Cyclo) at 100 μg/ml. When one compound was added after another, the time of addition of the second compound is indicated. All the incubations were done to have a contact of 30 min with lomofungin. A control of RNA and protein biosynthesis gave, respectively, 0.55 and 0.29 of the control level after 10 min of incubation with thiolutin and 0.93 and 0.19 after 10 min of incubation with cycloheximide.

incubation at 37 C were measured in 31 mutants. All but two were significantly impaired in RNA biosynthesis and much less in protein biosynthesis. One of the 29 that had the best growth at 22 C and a complete thermosensitivity at 34 C was further analyzed. After sporulation, six tetrads from this diploïd segregated 2^+:2ts, showing that it possessed a monogenic dominant mutation as expected. The diploïd formed by crossing one of the ts haploïds thus obtained with a haploïd of the opposite mating type bearing the ts dominant mutation obtained by lomofungin was induced to sporulate. The isolated spores germinated poorly, but 33 spores gave colonies all of which were thermosensitive. Thus the two mutations were allelic or closely linked to each other. This new allele has been named RNA 12-2.

Other similar selections gave more alleles of the same locus as well as at least two semidominant thermosensitive genes not allelic to the first gene or to each other. RNA biosynthesis was specifically inhibited at high temperature but not as strongly as with the first gene obtained. These two genes were not studied further.

Physiological study of the defect in the mutant RNA 12. The first physiological parameters measured were the kinetics of inhibition of RNA and protein synthesis after a shift to the high temperature. Figure 1 gives the results of a comparison of protein and RNA synthesis in the mutants and in the wild type. The rate of RNA synthesis was always retarded in our wild type for 1 h before reaching a new fast rate. This is not unique to our strain, since Warner and Udem (19) described the same effect. RNA synthesis in the mutant strain decreased to about one-tenth of the control rate but did not recover. In contrast to RNA synthesis, the rate of early protein synthesis was identical for the two strains but decreased slowly in the mutant to a virtual stop after 6 h. It appears therefore that the mutation specifically impairs RNA biosynthesis. The same experiment done with the heterozygote RNA 12-1/+ showed a 72% inhibition in the rate of RNA synthesis compared with the wild type after 90 min.

With the idea that one of the RNA polymerases could be modified in the mutant, the nature of the residual RNA synthesized at the high temperature was studied by two different

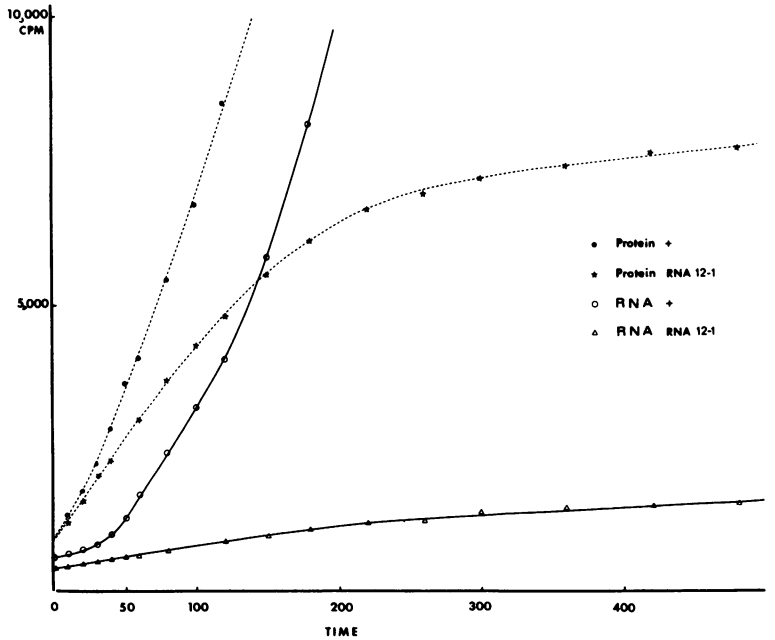

FIG. 1. *RNA 12-1 mutant strain and wild-type strain growing exponentially in minimal medium were preincubated during 40 min with either* L-[^{14}C]*leucine (20 μg, 0.1 μCi/ml) or* [^{14}C]*uracil (10 μg, 0.1 μCi/ml) to equilibrate the pools. At time zero, the cultures were shifted to 37 C and 0.5 ml of the cultures was removed at various times to estimate protein and RNA biosynthesis. The samples were added to 2 ml of 10% trichloracetic acid, equilibrated during at least 20 min in melting ice, and filtered on 0.45-μm membrane filters (Millipore Corp.). The dried filters were then counted with 10 ml of a toluene-5% 2,5-diphenyloxazole solution in an SL 30 Intertechnique spectrometer scintillation counter. The counts, not corrected for initial optical densities (which were comparable), were directly plotted as a function of the time of harvesting.*

approaches. The RNAs synthesized at high temperature in the mutant and in the wild type were examined in parallel sucrose gradients. Although the amount of the RNAs synthesized was much smaller in the mutant (about 1/20) than in the wild type, their natures were similar (Fig. 2). A relative increase in the heterogenous RNA class versus ribosomal RNA and transfer RNA was observed in the mutant. This increase has also been demonstrated by measuring the amount of polyuridylic acid-bound radioactivity, which was 9.2% of the total RNA in the mutant and 5.2% of the total RNA in the wild type. Nevertheless, this difference was not large enough to confirm the hypothesis that the mutant was impaired in RNA polymerase A, and we decided to measure RNA polymerase A and B activities directly.

Heat stability of the RNA polymerases activities. RNA polymerase B was purified to about 50% homogeneity from the RNA 12-1 mutant and from parental cells (FL 100) and then assayed at varying temperatures. No significant difference was found between the rates

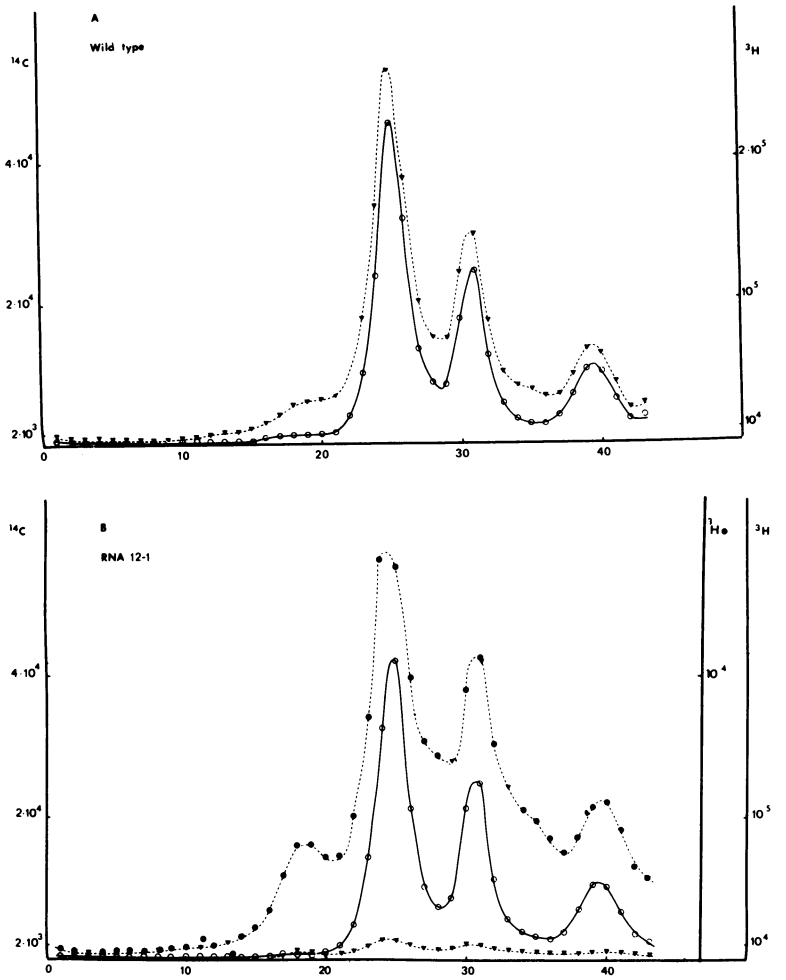

FIG. 2. *Twenty milliliters of cultures of the RNA 12-1 mutant strain and the wild-type strain growing in exponential phase at 22 C was labeled by 1 μCi of [^{14}C]cytosine. After 6 h the label was completely taken up from the medium due to the high affinity of the cytosine permease. The cultures were then shifted at 37 C, and after 70 min of incubation [^{3}H]uracil was added to the culture. After 30 min more the cultures were stopped by adding 2 volumes of cold ethanol. The RNA was then extracted and centrifuged on a sucrose gradient as previously described (13), and the fractions were counted for ^{3}H and ^{14}C radioactivity. The solid lines, which correspond to ^{14}C radioactivity, show the profile of stable RNAs made at 22 C in the two strains. The dashed lines, corresponding to ^{3}H radioactivity, indicate the nature and the amount of the RNAs synthesized during 30 min at the restrictive temperature. In (B), the ^{3}H counts were plotted directly or multiplied by a factor of 20 to visualize better the repartition of the different RNA species.*

of RNA synthesis catalyzed by the two polymerase B preparations (Fig. 3). With alkali-denatured DNA as template, the optimum temperature was between 28 to 37 C, whereas 50% of the maximal activity was obtained at 22 C (Fig. 3). With native DNA, the optimum temperature was 30 C, and the RNA polymerase B preparation from mutant cells showed the same temperature dependency curve as the control enzyme. Other allelic dominant mutants were tested for thermosensitive RNA polymerase B in the same way (RNA 12-4, and RNA 12-5). None of them exhibited an altered RNA polymerase B activity under the above assay conditions.

The effect of incubation temperature upon RNA polymerase A activity is shown in Fig. 4. The optimum temperature was around 25 C, although it seemed somewhat higher in the control. At 45 C a considerable percentage of activity remained (about 25%), suggesting that the enzyme from mutant cells is not particularly heat sensitive. Polymerase A from the three other mutants, RNA 12-3, RNA 12-4, and RNA 12-5, assayed in the same way behaved like the control enzyme A. The heat stabilities of purified RNA polymerase A from strain RNA 12-1 and FL 100 were compared at 46 C. Heat inactivation curves were similar. After 5 min of incubation at 46 C in the absence of DNA and substrates, 70% of the initial activity was lost in both cases. It should be stressed that the above results were obtained by using enzyme preparations at the same stage of purification [*O*-(diethylaminoethyl)-cellulose step] (2). Different results were obtained with RNA polymerase A preparations at an earlier stage of purification (phosphocellulose step) (2); for example, they showed a markedly lower optimum temperature. However, the control enzyme from parental cells exhibited the same shift in optimum temperature, which could reflect the presence of contaminating nucleases or proteolytic enzymes in the crude preparation.

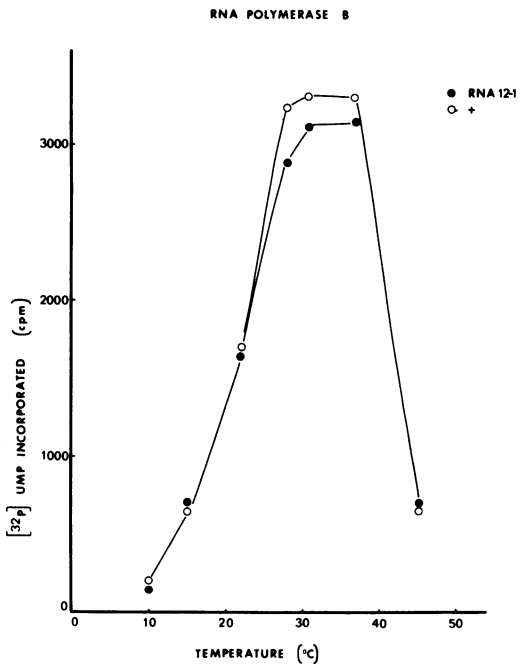

FIG. 3. *Effect of incubation temperature upon RNA polymerase B activity. RNA polymerase B was purified and assayed at different temperatures as described in the text, using 1.6 unit of enzyme (4) and denatured deoxyribonucleic acid as template. [α-^{32}P]uridine triphosphate was used as labeled nucleoside triphosphate (30,000 counts/min per nmol). [α-^{32}P]uridine triphosphate was used as labeled nucleoside triphosphate (30,000 counts/min per nmol). Symbols: (●) RNA polymerase B from RNA 12-1 cells; (○) RNA polymerase B purified from parental cells (FL 100).*

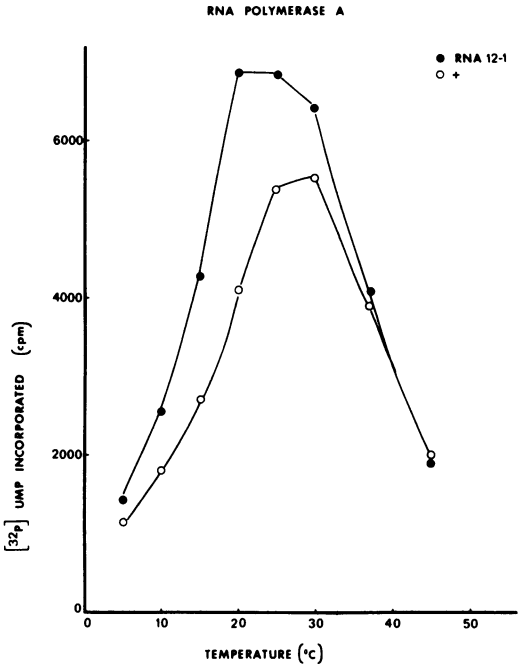

FIG. 4. *Effect of incubation temperature upon RNA polymerase A activity. RNA polymerase A was purified as described in the text and analyzed at different temperatures, using 1.5 unit of enzyme (2) and native deoxyribonucleic acid as template. [α-^{32}P]uridine triphosphate was used as labeled nucleoside triphosphate (56,000 counts/min per nmol). Symbols: (●) RNA polymerase A from RNA 12-1 cells; (○) RNA polymerase A purified from the parental cells (FL 100).*

Residual RNA synthesis in isolated nuclei. Since the RNA polymerases isolated from the

TABLE 2. *Biosynthesis of RNA in isolated nuclei*[a]

Determination	Strain			
	+	RNA 12-1	+	RNA 12-1
Temp and incubation time	22 C, 24 min	22 C, 24 min	37 C, 12 min	37 C, 12 min
Activity in counts/min per unit of initial optical density	10.7	8.3	9.2	9.8

[a] The conditions of nuclei isolation and of the measurement of their activity are described in Materials and Methods.

mutant and wild-type strains behaved identically, the possibility remained that a common cofactor lost during purification or not involved in the in vitro assays could be thermosensitive in the mutant. For this reason the residual RNA synthesis occurring in isolated nuclei was measured in the wild type and in the mutant according to the technique of Wintersberger et al. (20); they were also identical in this respect (Table 2).

DISCUSSION

In yeast, the use of thermosensitivity or resistance to inhibitors to obtain RNA polymerase mutants has not yet been fruitful. The different techniques we tried have failed to produce such a mutant. Nevertheless, the use of killing by lomofungin (which seems to occur specifically when RNA biosynthesis is active) allowed the selection of a thermosensitive mutant dominant over the wild-type allele and strongly inhibited for RNA biosynthesis. The dominance of a mutant such as this could have been explained very easily as for *E. coli* RNA polymerase mutants (9) by the fact that when a mixture of thermosensitive and wild-type RNA polymerase molecules transcribe one gene, retention of the thermosensitive molecules to the DNA after the shift to the high temperature prevents the normal enzymes from transcribing further. This effect would likely be more pronounced when RNA polymerases molecules are crowded along the genes. A single calculation of the in vivo rates of transcription in yeast gives about 31 RNA polymerase molecules for one ribosomal RNA cistron ($\simeq$6,200 + 2,500 nucleotides) and three RNA polymerase molecules for a ribosomal protein cistron of 1,200 nucleotides; this indicates a very similar spacing for the two kinds of transcription and suggests that the dominance effect must be similar for the different thermosensitive RNA polymerases. The in vitro assays have not furnished any evidence to strengthen the hypothesis of an RNA polymerase mutation. Nevertheless, it must be pointed out that a thermosensitive step occurring at the level of the initiation of transcription could be easily missed in the direct RNA polymerase assay as well as in the measurement of the residual RNA synthesis in nuclei for two reasons: in the first case the initiation seems different from in vivo initiation (5), and in the second case there is generally no new initiation at all. It is worthwhile noting that a thermosensitive mutation acting at the level of RNA chain initiation will be dominant provided that this mutation does not alter the affinity of the enzymatic complex for DNA promotors but impairs a later step in the process of initiation. Further work on this mutant will therefore await the avilability of an in vitro system in which the normal initiation of RNA transcription is measurable.

With our present knowledge we believe that the most likely explanation of the dominant behavior of the mutant is the thermosensitivity of a common cofactor for all RNA polymerases necessary for the normal initiation of RNA transcription in eukaryotic cells. Unlike the *E. coli* σ factor, this protein would not bind to the RNA polymerases before binding to the DNA but would bind to the DNA before binding to the RNA polymerases.

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