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Role of Isoleucyl-Transfer Ribonucleic Acid Synthetase in Ribonucleic Acid Synthesis and Enzyme Repression in Yeast

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Temperature-sensitive mutations in the isoleucyl-transfer ribonucleic acid (tRNA) synthetase of yeast, ilS$^-$1-1 and ilS$^-$1-2, were used to examine the role of aminoacyl-tRNA synthetase enzymes in the regulation of ribonucleic acid (RNA) synthesis and enzyme synthesis in a eucaryotic organism. At the permissive temperature, 70 to 100% of the intracellular isoleucyl-tRNA was charged in mutants carrying these mutations; at growth-limiting temperatures, less than 10% was charged with isoleucine. Other aminoacyl-tRNA molecules remained essentially fully charged under both conditions. Net protein and RNA syntheses were rapidly inhibited when the mutant was shifted from the permissive to the restrictive temperature. Most of the ribosomes remained in polyribosome structures at the restrictive temperature even though protein synthesis was strongly inhibited. Two of the enzymes of isoleucine biosynthesis, threonine deaminase and acetohydroxyacid synthetase, were derepressed about twofold during slow growth of the mutants at a growth-limiting temperature. This is about the same degree of derepression that is achieved by growth of an auxotroph on limiting isoleucine. We conclude that charged aminoacyl-tRNA is essential for RNA synthesis and for the multivalent repression of the isoleucine biosynthetic enzymes. Aminoacyl tRNA synthetase enzymes appear to play important regulatory roles in the cell physiology of eucaryotic organisms.

The aminoacyl-transfer ribonucleic acid (tRNA) synthetase enzymes of procaryotic cells play a key role in the regulation of cellular physiology (17). There is evidence that in order for an amino acid to exert its regulatory role upon ribonucleic acid (RNA) synthesis and the synthesis of its own biosynthetic enzymes, it must be attached to its respective tRNA molecule (3, 4). The availability of temperature-sensitive aminoacyl-tRNA synthetase mutants of *Saccharomyces cerevisiae* (7) allows an investigation of the role of aminoacyl-tRNA synthetase enzymes in the regulation of the eucaryotic cell. The experiments reported here examine the degree of intracellular charging of isoleucyl-tRNA at growth-restricting temperatures in temperature-sensitive yeast mutants carrying defective isoleucyl-tRNA synthetases and the consequences of uncharged isoleucyl-tRNA upon the cell's ability to synthesize RNA and repress the isoleucine biosynthetic enzymes.

MATERIALS AND METHODS

Strains and media. The origin of the parent strain A364A, and the derivation of the mutants, ts$^-$341 and ts$^-$443, from it by mutagenesis with nitrosoguanidine were described previously (6). These two mutant strains carry single temperature-sensitive lesions in the isoleucyl-tRNA synthetase structural gene (7), designated ilS$^-$1-1 and ilS$^-$1-2, respectively. Strain H 54-3-1 (α ilS$^-$1-1 is$_5^-$ur$_1^-$tr$_2^-$ad$_1^-$) was derived by standard genetic techniques from A364A and strain M12 ($\alpha$is$_5^-$tr$_2^-$). Cells were grown in one of the following media: YM-1 (6), YM-5 (6), YILV (12), minimal medium (12), or YM-1 + ILV (YM-1 containing 5 × 10^{-3}M L-isoleucine, L-valine, and L-leucine). In one experiment, an isoleucine requirement was supplied by L-isoleucylglycine (0.15 mg/ml). In this case the availability of isoleucine is rate-limiting, since the doubling time increases from 2 to 3 hr in the presence of isoleucine to 5 to 7 hr in the presence of isoleucylglycine.

Degree of tRNA charging. Cells growing at a concentration of less than 10^7 cells/ml at 23 C were concentrated by centrifugation, suspended in YM-5 at a concentration of 2 × 10^8 cells/ml, and incubated

for 15 min at 23 C. Cultures were then shifted to 36 C and incubated for various periods of time. Samples of 50 ml were collected by the addition of 0.01 volume of 3% SDS (sodium dodecyl sulfate) followed immediately by an equal volume of 80% phenol. The mixture was shaken vigorously at 23 C for 5 min, the phases were separated by centrifugation, and the aqueous layer was removed. The aqueous layer was extracted twice with 2 volumes of ether, and 0.1 volume of 20% potassium acetate (*p*H 5.2). Two volumes of cold ethyl alcohol was added, and the mixture was left at −20 C for 30 min. The precipitate was collected by centrifugation and dissolved in 5 ml of 0.1 M potassium acetate (*p*H 5.2). A 3.5-ml portion of the charged tRNA sample was treated with 1.0 ml of 0.05 M $NaIO_4$ at room temperature in the dark for 30 min. The decomposition of the periodate was followed at 238 nm. If necessary, 0.5 ml of 0.05 M $NaIO_4$ was added to maintain the absorbancy at 238 nm. A twofold molar excess of glucose (relative to the $NaIO_4$) was added, and the sample was dialyzed extensively against distilled water at 4 C. During the first 2 hr, NH_4OH was added to adjust the *p*H of the water to 10. The control sample was dialyzed but was not treated with periodate. The samples were lyophilyzed, and the acceptor activity of the tRNA was determined in the usual assay mixture (7) by using saturating amounts of a partially purified activating enzyme preparation that was essentially free of tRNA added in saturating amounts. For each result, the acceptor activity of a tRNA preparation was determined both before and after periodate oxidation with at least three different concentrations of tRNA. The result is expressed as: percentage of tRNA charged = acceptor activity of periodate-treated tRNA ÷ acceptor activity of untreated tRNA × 100.

Macromolecule synthesis and sucrose gradient analysis. The net increase in protein, RNA, and deoxyribonucleic acid (DNA) and the incorporation of radioactivity into these macromolecular components were determined as described previously (6). Sucrose density gradient analyses of RNA components (9) and polyribosomes (8) were also described previously.

Enzyme assays. Threonine deaminase (13) and acetohydroxyacid synthetase (14) were assayed in permeabilized cells (15) as previously described.

RESULTS

Degree of tRNA charging. The isoleucyl-tRNA synthetase activity in extracts of mutants ts⁻341 and ts⁻443 has been shown to be thermolabile relative to the same activity in parent extracts (7). Genetic evidence indicates that the mutation producing the temperature-sensitive growth response in these mutants is closely linked to and probably identical with the mutation causing the defective isoleucyl-tRNA synthetase detected in vitro. This mutation has been mapped on chromosome II and the locus has been designated I1S (11). The expression of this defect in vivo would be expected to result in a discharging of the isoleucyl-tRNA. The following experiment verifies this expectation. Cultures of mutants ts⁻341, ts⁻443, and the parental strain growing at 23 C, the permissive temperature, were shifted to 36 C, the restrictive temperature, and the degree of charging of intracellular tRNA was determined by a procedure which instantaneously inactivates cellular processes and allows the extraction of the tRNA from the cell. The degree of charging of phenylalanyl, valyl, and isoleucyl-tRNA remains above 70% in the parent culture at all times (Fig. 1). In the mutants, phenylalanyl and valyl-tRNA are nearly 100% charged after the shift to 36 C, whereas the level of charged isoleucyl-tRNA dropped from 70 to 100% at 23 C to less than 10% after the shift to 36 C.

Macromolecule synthesis. The net increase in protein, RNA, and DNA in cultures of mutant ts⁻341 growing at the permissive temperature and following a shift to the restrictive temperature was compared with that in cultures of the parent strain (Fig. 2). The mutant grows slightly slower than the parent strain at 23 C, exhibiting an exponential increase in macromolecular components with a doubling time of about 3.3 hr. At 36 C, the parent strain grows slightly faster than at 23 C, whereas the mutant exhibits a strong inhibition of macromolecule synthesis. Protein and DNA increase approximately 30% in the mutant after 5 hr at 36 C, and RNA increases only about 10%.

The kinetics of incorporation of precursors into protein and RNA in both mutants and the parent strain was examined after a shift to 36 C (Fig. 3). As expected from the previous result, protein and RNA syntheses were both strongly inhibited relative to that of the parent strain. Thus, it may

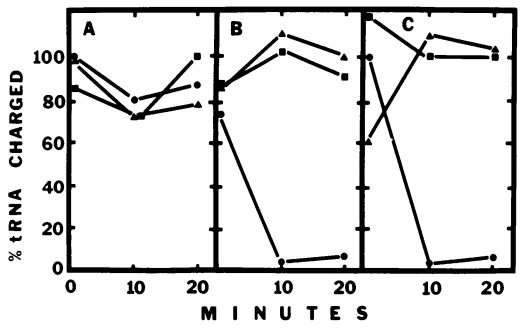

FIG. 1. *Degree of tRNA charging in the parent strain and mutants ts⁻341 and ts⁻443 after a shift to 36 C. Cultures of the parent strain (A) and mutants ts⁻341 (B) and ts⁻443 (C) growing in YM-5 medium at 23 C were shifted to 36 C and processed as described. The degree of intracellular tRNA charging was determined for phenylalanyl (■), valyl (▲), and isoleucyl (●) tRNA.*

be concluded that both protein and RNA syntheses are dependent upon the presence of an active isoleucyl-tRNA synthetase.

Polyribosome stability. It has been previously noted that a variety of defects inhibiting polypeptide chain elongation result in a stabilization of polyribosome structures in yeast (8). This phenomenon is further verified by the behavior of polyribosomes in mutants ts⁻341 and ts⁻443 (Fig. 4). In mutant ts⁻341, the percentage of ribosomes which were in polyribosomes dropped from 80% at 23 C to approximately 70% after the shift to 36 C and remained essentially constant. Mutant ts⁻443 also contained 80% of the ribosomes as polyribosomes at 23 C; this figure dropped to approximately 50% after the shift to 36 C. These values are to be compared with the parent strain in which the polysome level drops from 90% before to 80% after the shift. Thus, the decay of polyribosomes to monoribosomes in the two mutants at the restrictive temperature is small and not commensurate with the decay of protein synthetic capacity.

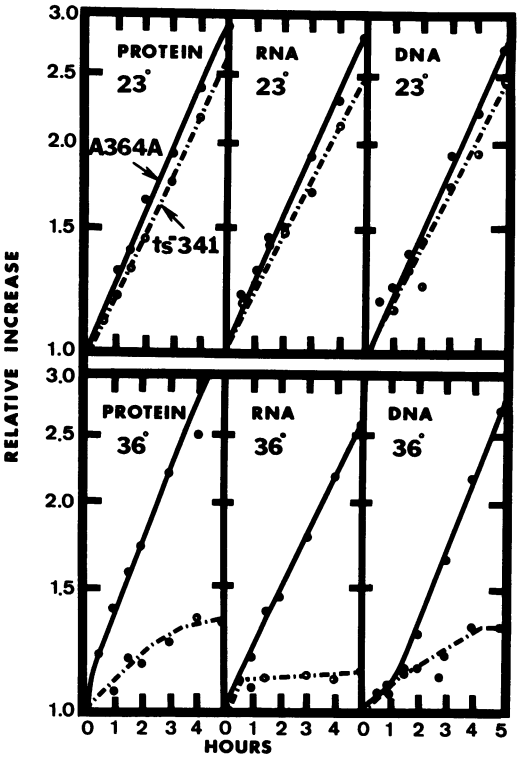

FIG. 2. *Relative increase in protein, RNA, and DNA in cultures of the parent strain and mutant ts⁻341 at 23 and 36 C. Cultures of the parent strain (●) and mutant ts⁻341 (○) were grown for at least six generations at 23 C in YM-5 medium plus ¹⁴C-adenine (0.1 μc/ml) and ³H-lysine (0.3 μc/ml) to uniformly label the nucleic acids with ¹⁴C and the protein with ³H. (These strains are adenine and lysine auxotrophs.) At time zero, one-half of each culture was shifted to 36 C, and samples were removed at various times to determine the radioactivity incorporated into each of the macromolecules. The data are plotted as the radioactivity in each component divided by the radioactivity in that component at zero time.*

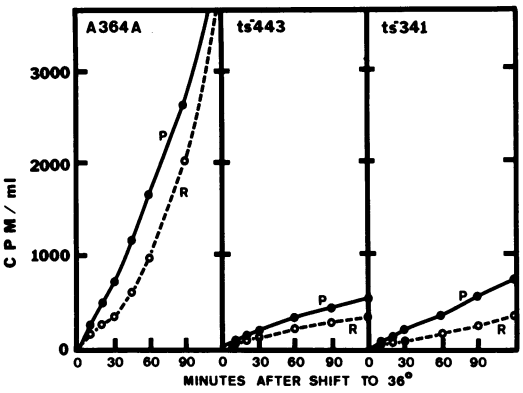

FIG. 3. *Kinetics of protein and RNA synthesis in the parent strain and mutants ts⁻341 and ts⁻443 after a shift to 36 C. Cultures of cells growing at 23 C in YM-5 medium were shifted to 36 C, and ¹⁴C-adenine (0.035 μc/ml) or reconstituted ¹⁴C-protein hydrolysate (0.1 μc/ml) was added. One-milliliter samples were removed at various times and analyzed for radioactivity incorporated into macromolecules.*

RNA components synthesized at the restrictive temperature. Although the incorporation of radioactive precursors into RNA is strongly inhibited in mutant cultures at the restrictive temperature, some RNA is synthesized. It is of interest to know whether this synthesis represents a special class of RNA molecules. To examine this question, mutant ts⁻443 was allowed to incorporate radioactivity into RNA for 2 hr at 23 C and at 36 C, and the RNA components were then fractionated by sucrose density-gradient centrifugation (Fig. 5). The optical density profile which exhibits two peaks corresponding to the two ribosomal RNA components is coincident with the radioactivity profile obtained from the culture incubated at 23 C; in addition, a small peak of radioactivity at the top of the gradient demonstrates the formation of transfer RNA. The radioactive profile from the culture incubated at 36 C displays the same three peaks, indicating that, although the extent of synthesis is much less, some ribosomal RNA is synthesized at the restrictive temperature. The amount of low molecular weight RNA which sediments in the position expected for tRNA is proportionately larger in this culture. Essentially identical results were obtained with cultures of mutant ts⁻341.

Regulation of the isoleucine biosynthetic enzymes. The synthesis of the isoleucine biosynthetic enzymes in yeast is regulated via multivalent repression by the presence or absence of isoleucine, valine, and leucine in the culture medium (1, 12). It was of interest, therefore, to determine whether isoleucine was itself the regulator or whether isoleucine had to be activated by isoleucyl-tRNA synthetase to exert its regulatory effect. This question could be examined by measuring the specific activity of the isoleucine biosynthetic enzymes from cells grown in a medium containing a high isoleucine concentration but under conditions in which the charging of isoleucine onto tRNA was the growth-limiting reaction. This condition is achieved with mutant ts⁻341 at growth-limiting temperatures. In order to achieve maximal sensitivity in the measurements to be made, an intermediate temperature was sought at which considerable growth could be achieved but which would still be growth-limiting. Figure 6 shows the rate of protein synthesis in the parent strain and the mutant at various temperatures. The parent strain, as expected, synthesizes protein more rapidly at 32 and 37 C than it does at 23 C. In contrast, mutant ts⁻341 synthesizes protein more slowly at all temperatures above 32 C than it does at 23 C; 34 C provides a nice compromise for the mutant, since considerable growth can be achieved. However, this growth is clearly limited by the temperature-sensitive mutation, since it occurs at only about one-half the 23 C rate.

Cultures of mutant ts⁻341 and the parent strain were grown at 23 and at 34 C in a variety

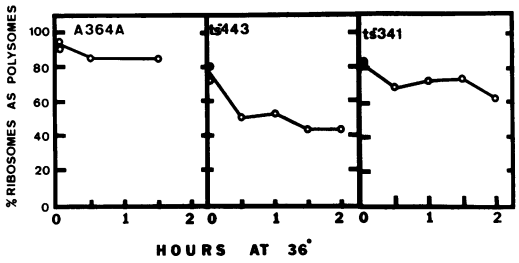

FIG. 4. *Percentage of ribosomes present as polyribosomes in cultures of the parent strain and mutants ts⁻341 and ts⁻443 after a shift to 36 C. Spheroplasts of the parent strain and mutants ts⁻341 and ts⁻443 growing at 23 C in YM-5 medium containing 0.4 M $MgSO_4$ were shifted to 36 C and collected at various times. Cytoplasmic extracts were displayed on sucrose gradients, and the percentage of the ribosomal optical density at 260 nm sedimenting faster than the monoribosome peak was determined. The data are from several experiments.*

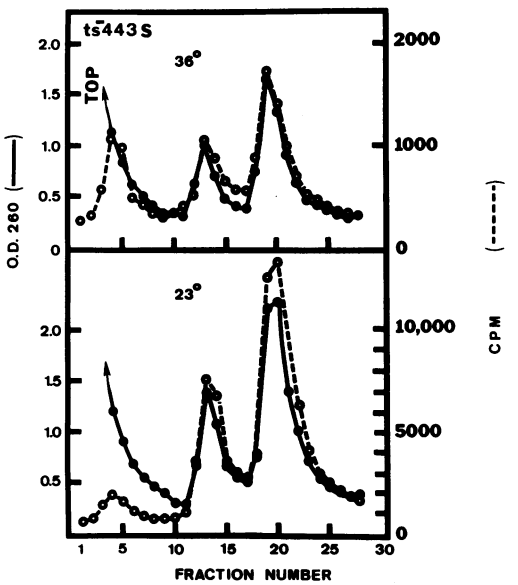

FIG. 5. *Sedimentation profile of RNA synthesized at 23 C and 36 C in mutant ts⁻443. Spheroplasts of mutant ts⁻443 in YM-5 medium containing 0.4 M $MgSO_4$ at 23 C were left at 23 C or shifted to 36 C and ^{14}C-adenine (0.2 μc/ml) was added. After 2 hr of incorporation, the cultures were collected, the cells were lysed, and their contents were layered on a 5 to 20% sucrose gradient. Centrifugation was for 15 hr at 80,000 × g in a Spinco SW25 head. Fractions were collected by pumping the contents out of the top of the tube and monitored for optical density at 260 nm and radioactivity. Note change of scale between 23 and 36 C cultures.*

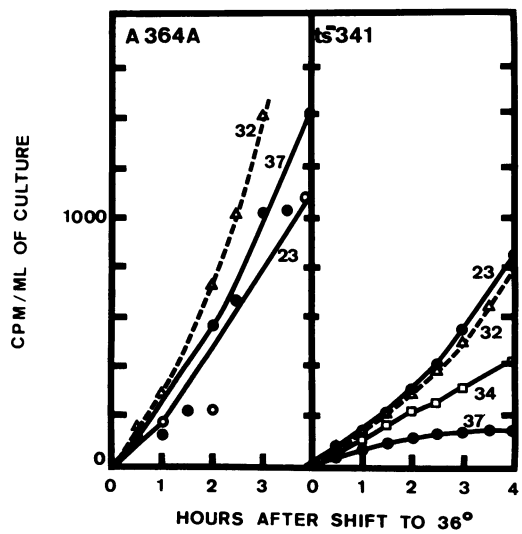

FIG. 6. *Protein synthesis at various temperatures in cultures of the parent strain and mutant ts⁻341. Cultures growing at 23 C in YM-5 medium were shifted to various temperatures, and reconstituted ^{14}C-protein hydrolysate (0.02 μc/ml) was added. Samples were removed at various times and analyzed for radioactivity incorporated into protein.*

of media containing high concentrations of isoleucine, and the specific activities of two enzymes in the isoleucine biosynthetic pathway were determined (Table 1). The specific activity of threonine deaminase is slightly less in the parent strain grown at 34 C than at 23 C; the mutant, on the contrary, exhibited a higher specific activity when grown at 34 C. The ratio of the activity at 34 C divided by the activity at 23 C is approximately twofold higher in the mutant than in the parent strain. The same effect is seen in the specific activity measurements of acetohydroxyacid synthetase. The magnitude of the effect was about the same on all the media tested. Thus, the repression exerted by isoleucine upon the activities of these two enzymes is less effective when the activity of isoleucyl-tRNA synthetase is inhibited.

TABLE 1. *Specific activity of threonine deaminase and acetohydroxyacid synthetase in the parent strain and mutant ts⁻341 growing at 23 and 34 C*[a]

Strain	Enzyme	Medium	Enzyme activity[b]		Ratio[c] (34/23 C)
			23 C	34 C	
A364A	TD	YM-5	2.30	1.80	0.78
		YM-5	2.18	1.44	0.66
		YILV	2.35	1.54	0.65
		YM-1 + ILV	1.68	1.11	0.66
		YM-1 + ILV	1.56	1.84	1.18
	AS	YM-5	0.133	0.122	0.92
		YM-5	0.107	0.097	0.91
		YILV	0.103	0.103	1.00
		YM-1 + ILV	0.063	0.065	1.03
		YM-1 + ILV	0.058	0.030	0.52
ts⁻341	TD	YM-5	2.50	2.99	1.20
		YM-5	1.41	2.46	1.75
		YILV	1.58	2.82	1.78
	AS	YM-5	0.095	0.291	3.06
		YM-5	0.070	0.142	2.03
		YILV	0.035	0.086	2.45
H54-3-1	TD	YM-1 + ILV	1.49	3.03	2.02
		YM-1 + ILV	3.45	4.90	1.42
		YM-1 + ILV	2.83	3.94	1.49
		YM-1 + ILV	2.11	4.03	1.91
		YM-1 + ILV	2.50	4.00	1.60
	AS	YM-1 + ILV	0.100	0.227	2.27
		YM-1 + ILV	0.134	0.408	2.98
		YM-1 + ILV	0.147	0.357	2.43
		YM-1 + ILV	0.177	0.309	1.75
		YM-1 + ILV	0.140	0.325	2.32

[a] The cells were grown overnight with shaking in the indicated medium at 23 C; the culture was then split. One portion was left at 23 C, and the other was transferred to 34 C. The cells were harvested after the 34 C cultures had quadrupled in turbidity as monitored at 650 nm.

[b] Expressed as micromoles of product in 20 min per mg of protein.

[c] Average ratios were as follows. Strain A364A: TD enzyme, 0.78; AS enzyme, 0.88. Strain ts⁻341: TD enzyme, 1.58; AS enzyme, 2.51. Strain H54-3-1: TD enzyme, 1.69; AS enzyme, 2.35.

TABLE 2. *Specific activity of threonine deaminase and acetohydroxyacid synthetase in strain H54-3-1 growing on saturating and limiting concentrations of L-isoleucine*[a]

Enzyme	Enzyme activity[b]		Ratio[c]
	Isoleucine	Isoleucylglycine	
TD	2.92	3.95	1.35
	1.60	5.10	3.19
AS	0.269	0.459	1.71
	0.229	0.544	2.37

[a] The cells were inoculated from a YM-1 preculture into the appropriate medium at 23 C. They were allowed to grow for at least two generations before harvesting.

[b] Expressed as micromoles of product in 20 min per mg of protein.

[c] Average ratios: enzyme TD, 2.27; enzyme AS, 2.04.

It is of interest to determine whether the extent of the specific activity change in these experiments (two- to threefold) is as great as that which can be achieved by isoleucine starvation. This point was tested by constructing a strain, H54-3-1, carrying the i1S⁻1-1 mutation and is_5^-, a mutation which inactivates the α-hydroxy-β-keto acid reductoisomerase (10) and confers isoleucine-valine auxotrophy. This strain was examined for threonine deaminase activity and for acetohydroxy acid synthetase activity after growth either at growth-limiting temperature (34 C, Table 1) or under conditions of isoleucine limitation (on isoleucylglycine, Table 2). It can be seen that the extent of derepression for the two enzymes was similar, whether the derepression was attained by temperature limitation or isoleucine limitation. We conclude that the regulatory effect exerted by isoleucine on the specific activities of its biosynthetic enzymes requires the activation of isoleucine.

DISCUSSION

The experiments reported here demonstrate that the activity of isoleucyl-tRNA synthetase is essential for RNA synthesis and for multivalent repression of the isoleucine biosynthetic enzymes in a eucaryotic organism, *S. cerevisiae*. This suggests that free isoleucine is not the regulator of RNA and enzyme synthesis but that the true regulator is a product of the reaction catalyzed by isoleucyl-tRNA synthetase. A likely candidate

is isoleucyl-tRNA; however, it is conceivable that some other reaction is catalyzed by this enzyme and that the regulatory metabolite is either the product of this second reaction or a metabolic product of isoleucyl-tRNA. The behavior of a mutant of *Neurospora crassa*, another eucaryote which accumulates arginosuccinate, has been interpreted to indicate that arginyl-tRNA formation is essential for repression of the arginine biosynthetic enzymes (16).

Although there is no conclusive evidence in any system for the role of tRNA in multivalent repression, our results and those of others (2, 3, 5) are highly suggestive.

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