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Induction of ornithine decarboxylase activity in a temperature-sensitive cell cycle mutant of Chinese hamster cells

(somatic cell genetics/fibroblasts)

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ABSTRACT **We have investigated the induction of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) activity in a temperature-sensitive cell cycle mutant of Chinese hamster fibroblasts. This activity is not induced at the nonpermissive temperature, although the synthesis of the majority of proteins is normal. From a combination of studies with inhibitors of mRNA synthesis and maturation (α-amanitin and cordycepin) and of protein synthesis (cycloheximide, diphtheria toxin, and emetine), we conclude that the temperature-sensitive block is at the level of translation of one or more specific mRNAs.**

Among the collection of temperature-sensitive (ts) mutants of mammalian cells reported there have been several cell cycle mutants which appear to have a defect in the G_1 phase of the cell cycle. As a result of such a defect they cannot initiate a round of DNA synthesis at the nonpermissive temperature (1–6). Biochemical studies on several of these mutants have revealed interesting differences between mutant and parental cells, but in none of the cases has the defect been traced to a single protein or biochemical reaction (6–12). An understanding of the events in the G_1 phase of the cell cycle is essential to our understanding of the control of the cell cycle and the control of proliferation of mammalian cells in general.

During the G_1 phase, ornithine decarboxylase (Orn decarboxylase; L-ornithine carboxy-lyase, EC 4.1.17) increases dramatically and reaches a relatively sharp maximum at or before the onset of DNA synthesis (13, 14). It is the rate-limiting enzyme in the pathway of polyamine biosynthesis (15), and in the absence of putrescine in the medium it is an essential enzyme for cell proliferation (16, 17). Orn decarboxylase activity is characteristically associated with rapidly proliferating cells in culture (18–21), tissues such as tumors (22), and regenerating rat liver (22, 23). The enzyme has one of the shortest half-lives of many enzymes studied (19, 21). The control of this activity is complex, and may involve translational control (24), a specific protein inhibitor (25–27), interconversion of multiple forms of the enzyme (28), rapid degradation, or feedback inhibition by the polyamines (15).

In this communication we present results of studies of the activity of Orn decarboxylase in a ts cell cycle mutant. We show that whereas the enzyme is induced in synchronized cultures at the permissive temperature, the enzyme activity is not induced in synchronized mutant cells placed at the nonpermissive temperature. We also present evidence for the existence of a temperature-sensitive defect at the level of translation of the mRNA for this enzyme.

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MATERIALS AND METHODS

Cell Cultures. The ts mutant and its wild-type parent are Chinese hamster fibroblasts that have been described (6). They were routinely maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 5% fetal calf serum (Irvine Scientific) in a 34° water-jacketed incubator flushed with 10% CO_2/air. For the temperature shift experiments we used a 40.8° water-jacketed incubator kept in a 37° room or a 40.8° water bath.

Synchronization by Serum Starvation. The procedure has been described (6), except that cells were released from the block by the addition of fresh modified Eagle's medium containing only 5% fetal calf serum.

DNA Synthesis. Incorporation of radioactive thymidine (dThd) into DNA was measured as described (6).

Analysis of Proteins. Wild-type or mutant cells released from serum starvation and placed at either 34° or 40.8° were incubated at various times in minimum essential Eagle's medium containing 5% fetal calf serum, 1/20th of the normal amount of methionine, and 50 μCi of [^{35}S]methionine per ml, for 2 hr. Protein was collected by the procedure of Milman *et al.* (29), and two-dimensional gel electrophoresis was carried out according to the modified method of O'Farrell (30). Fluorography was performed as described by Bonner and Laskey (31).

Assay of Orn Decarboxylase. Cells were washed, collected by scraping, and lysed by three freeze-thaw cycles. Reaction buffer (0.5 ml) containing 0.2 mM EDTA, 5 mM dithiothreitol, and 50 μM pyridoxal phosphate in 50 mM Tris·HCl, pH 7.1, was added and the samples were centrifuged at 10,000 rpm for 10 min in a Sorvall RC2-B centrifuge. Duplicate samples of supernatant (0.15 ml) were placed in plastic test tubes. After addition of 0.5 μCi of [1-^{14}C]ornithine (specific activity 45 Ci/mol), the tubes were capped with serum stoppers holding a polyethylene well (Kontes) and the samples were incubated at 37° for 1 hr. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid through the stopper by means of a syringe, and 0.2 ml of 0.2 M NaOH was added to the well by similar means to trap the $^{14}CO_2$ released in the reaction. The radioactivity was measured in a Triton/toluene scintillation fluid (32). Protein was determined by the method of Lowry *et al.* (33).

Isotopes and Chemicals. [^{3}H]dThd, [1-^{14}C]ornithine·HCl, and [^{35}S]methionine were obtained from New England Nuclear. Cordycepin and emetine·HCl were obtained from Sigma, α-amanitin from Boehringer Mannheim, pyridoxal phosphate from Aldrich, cycloheximide from Calbiochem, and diphtheria toxin was provided by R. Draper (University of California San Diego, Dept. of Biology).

Abbreviations: ts, temperature sensitive; Orn decarboxylase, ornithine decarboxylase.

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RESULTS

Macromolecular Synthesis. It has been established (6) that in mutant cells synchronized by mitotic selection or serum deprivation DNA synthesis is not initiated at the nonpermissive temperature (40.8°). For comparison with data to follow, the results of another such experiment are shown in Fig. 1. It has also been shown (6) that total RNA and total protein synthesis rates are not altered in the mutant at 40.8° during the first 10 hr after the release from serum starvation, but a more detailed analysis of protein synthesis seemed desirable. Fig. 2 shows typical autoradiograms of proteins from pulse-labeled cells that were separated by two-dimensional electrophoresis on polyacrylamide gels. Most of the same spots are seen when mutant cells were labeled either at 34° or at 40.8°, and no striking differences between the two temperatures are seen when pulse labeling is carried out at various times during the G_1 phase. Similarly, the majority of the spots found in the mutant autoradiograms are also seen in the patterns obtained from wild-type cells labeled at 34° or 40.8°. There are some missing spots and differences in spot size which remain to be interpreted but the conclusion is that there is no general defect in the synthesis of the major proteins in the mutant at the nonpermissive temperature.

Orn Decarboxylase. In an attempt to determine how long before the onset of DNA synthesis the ts defect could affect reactions in the cells at 40.8°, we chose Orn decarboxylase activity as a readily measurable cell cycle marker. Fig. 3 shows the activity of this enzyme in synchronized wild-type and mutant cells at 34° and 40.8°. In wild-type cells at the permissive temperature the peak of activity occurs at 8 hr. For unexplained reasons, the activity in wild-type cells at 40.8° peaks earlier and is somewhat reduced. Since the wild-type cells grow more rapidly at 40.8° (6), the Orn decarboxylase activity might be expected to occur earlier, as observed. In contrast, the activity is almost totally absent in the mutant cells at 40.8°, while at 34° there is a 5- to 10-fold stimulation with a maximum at 9–10 hr. We do not yet know whether there is also a difference in the size of polyamine pools or in the rate of secretion of putrescine at the two temperatures.

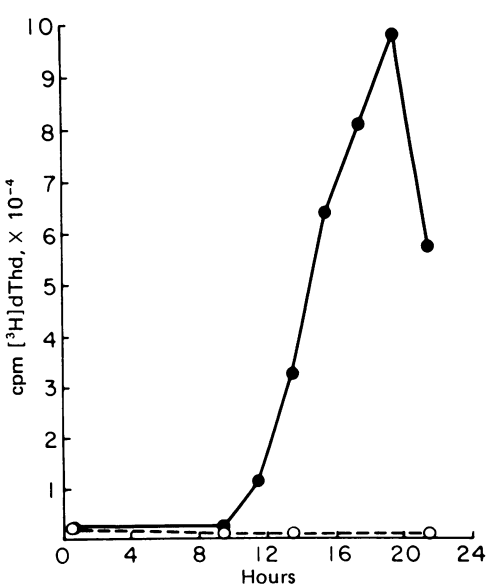

FIG. 1. DNA synthesis in cells synchronized by serum starvation. At zero time, fresh medium and serum were added to identical starved cultures of cells. Half of the plates were shifted immediately to 40.8°, the others left at 34°. Duplicate plates were labeled for 30 min with 1 μCi of [³H]dThd per ml (specific activity 55.5 μCi/mmol). Incorporation of label into DNA was determined, and the results are plotted at the midpoints of each pulse. ●, 34°; ○, 40.8°.

Several simple explanations for the lack of Orn decarboxylase activity in mutant cells at 40.8° have been ruled out. The enzyme itself is not temperature sensitive. All *in vitro* assays were carried out at 37°, but extracts from mutant cells grown at 34°, when placed at 40.8° for various lengths of time, did not lose Orn decarboxylase activity for at least 2 hr. We also examined whether there was a soluble inhibitor for the enzyme being produced at 40.8°. Mixing experiments with extracts from cells grown at 34° or 40.8° showed no evidence for such an inhibitor (results not shown).

Control at Transcriptional or Post-Transcriptional Level. The next series of experiments were aimed at finding out whether in the mutant at 40.8° there was a block in the production of mRNA for Orn decarboxylase and whether the defect was in the translation of this mRNA or in a post-translational modification step. At this time all such experiments rely heavily on the specificity of several well-known inhibitors. In Fig. 4*A* we first show that when mutant cells are shifted from 40.8° to 34° at 8 hr after the stimulation by serum, an induction of enzyme activity is clearly observed, with a maximum at around 10 hr. In a similar experiment (Fig. 4*B*) α-amanitin was added to identical aliquots of mutant cells at 40.8° at various times, and this inhibitor of RNA polymerase II (34) was left in the medium when the cells were shifted back to the permissive temperature at 8 hr. When α-amanitin was added at zero hour, no activity was observed, but noticeable activity appeared when the drug was added as early as 2 hr after the serum. The amount of activity increased in proportion to the length of time the cells spent in the absence of the drug, and superinduction occurred when the drug was added at later times. We have made related observations with actinomycin D (unpublished). At this time we do not wish to speculate further on the mechanism of superinduction, but we would like to conclude that mRNA or a precursor is produced at 40.8°.

One may next ask whether the defect is in the post-transcriptional processing of the mRNA precursors and in the export to the cytoplasm. To address this question we used cordycepin as an inhibitor of polyadenylylation (35) in an experiment analogous to that described in Fig. 4*B*. Again the drug was added at various times to cells at 40.8°, and the inhibitor remained present after the downshift. The results (Fig. 4*C*) indicate that an amount of enzyme activity is induced which depends on the length of time that the cells have spent at 40.8° in the absence of the drug, and we conclude that the mRNA is in fact polyadenylated.

To ascertain whether *de novo* protein synthesis is necessary for the appearance of Orn decarboxylase activity after the shift from 40.8° to 34°, experiments were performed in which inhibitors of protein synthesis were added to the cells 0.5 hr before or at the time of the downshift. As shown in Fig. 4*D*, cycloheximide or diphtheria toxin is completely effective in blocking the appearance of enzyme activity. Emetine had a similar effect (results not shown).

The final experiments presented here were aimed at learning something about the turnover rates of Orn decarboxylase in wild-type and mutant cells at 34° and 40.8°. In these experiments the enzyme activity was induced at 34°. Two hours before the peak of activity, cycloheximide was added to some aliquots of cells, and aliquots of cells with or without the drug were transferred to 40.8°. As shown in Fig. 5, the enzyme activity disappears rapidly in wild-type and mutant cells in the presence of cycloheximide, regardless of the temperature. In mutant cells shifted to 40.8° without cycloheximide, Orn decarboxylase activity declined rapidly, while in wild-type cells the enzyme activity remained elevated for some time. In the mutant the temperature shift has the same effect as addition of the protein synthesis inhibitor. It thus appears that no new

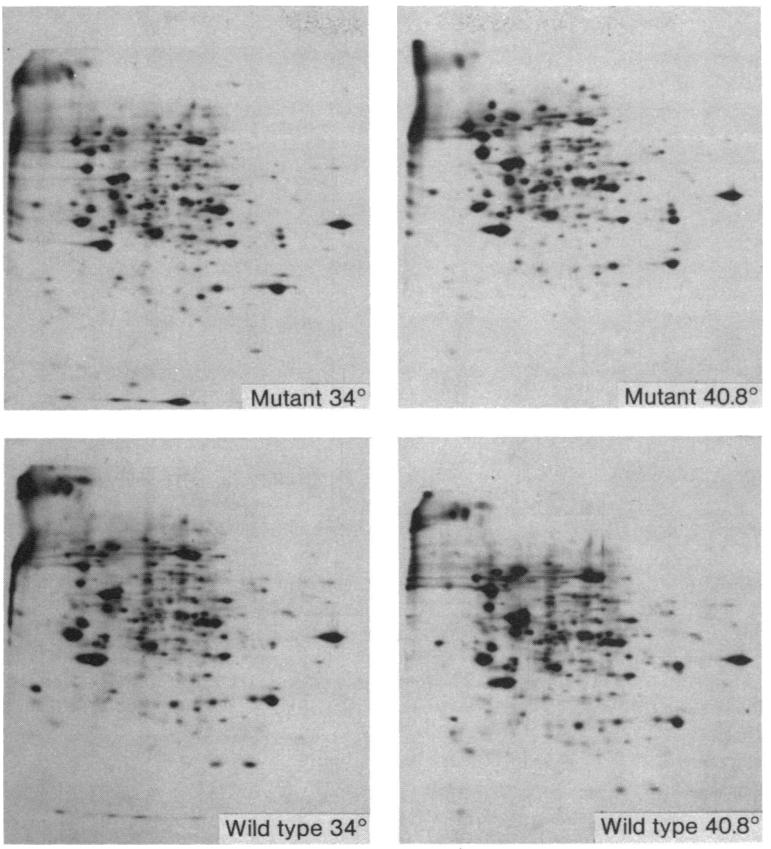

FIG. 2. Autoradiograms of proteins on two-dimensional acrylamide gels. Cells were synchronized by serum starvation and labeled between 9 and 11 hr (at 34°) and between 5 and 7 hr (at 40.8°) with 50 μCi of [^{35}S]methionine per ml (specific activity 567 μCi/mmol). Cells were harvested and protein solutions were prepared. Electrophoresis was according to the modified procedure of O'Farrell (30); 10^5 cpm were loaded onto the gel; exposure to x-ray film was for 1 day. Isoelectric focusing was carried out in the horizontal dimension using ampholines in the pH range 3.5–10.0. Acidic proteins are on the left of the figures.

protein is being made in mutant cells placed at 40.8°, and that the arrest is very rapid.

DISCUSSION

A straightforward interpretation of the results presented here is the following: At the nonpermissive temperature in the ts mutant, mRNA for Orn decarboxylase is produced, but there is a specific block in the translation of this message. This interpretation depends on the specificity of the drugs used and on the speed at which these drugs enter the cell and become effective.

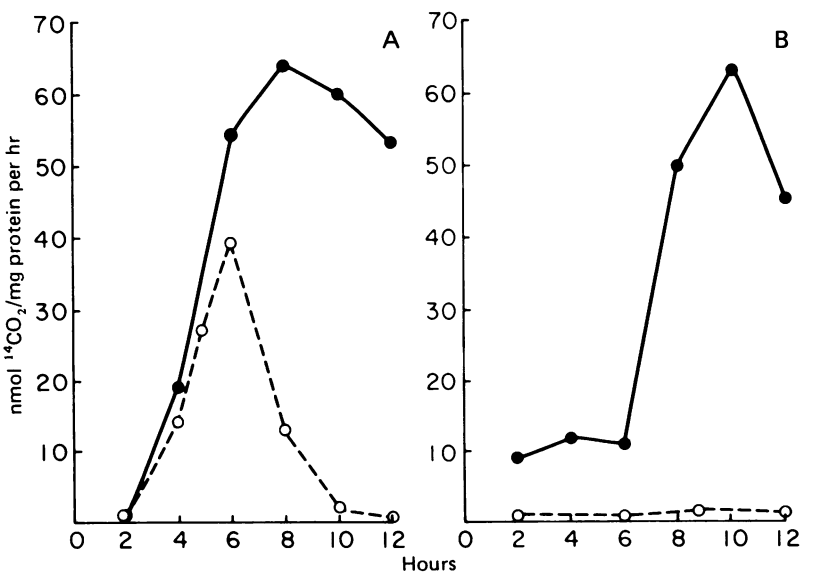

FIG. 3. Orn decarboxylase activity in wild-type (*A*) and mutant (*B*) cells. Cells were synchronized by serum starvation and addition of serum at 0 time. Aliquots of cells were placed at 40.8°. At the indicated times, aliquot plates were removed and enzyme activity was measured at 37°. ●, Activity in cells at 34°; ○, activity in cells at 40.8°.

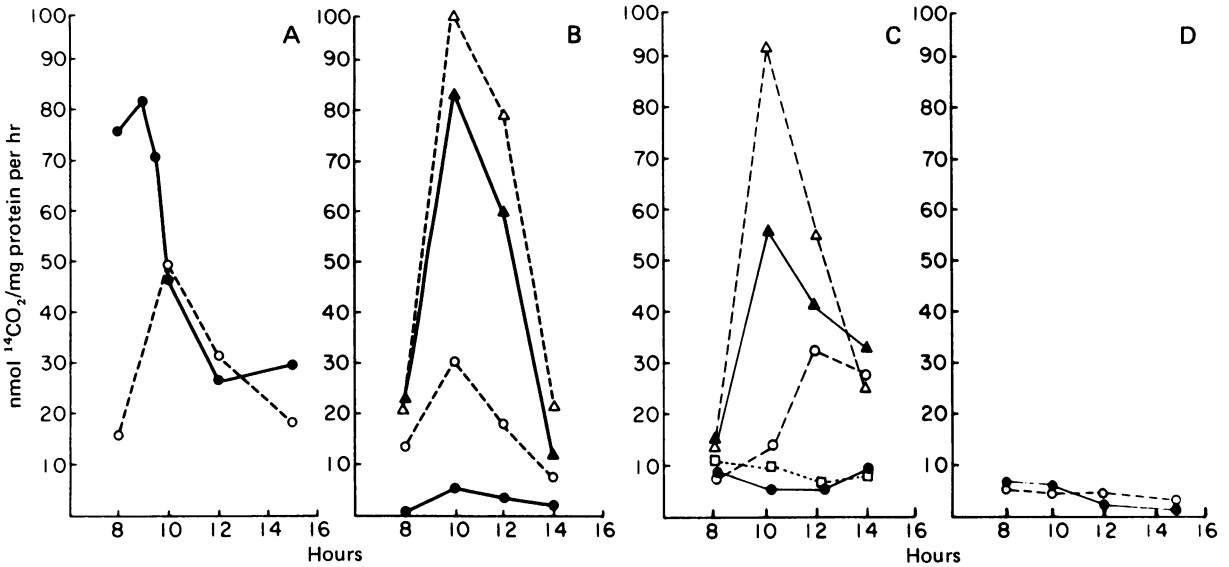

FIG. 4. Effect of drugs on induction of Orn decarboxylase. (*A*) Synchronized mutant cells were either left at 34° (●) or placed at 40.8° at zero time. After 8 hr these plates were shifted to 34° (○). Orn decarboxylase activity was determined. (*B*) Same as in *A*, except that α-amanitin (10 μg/ml) was added to the plates initially at 40.8° at 0 (●), 2 (○), 4 (▲), and 6 (△) hr. The drug remained in the medium throughout the period after the shiftdown at 8 hr. (*C*) Same as in *B*, except that cordycepin (25 μg/ml) was added to the plates at 40.8° at 0 (●), 2 (○), 4 (▲), and 6 (△) hr. The drug remained in the medium after the shiftdown. 40.8° control, no shift to 34° or drug added (□). (*D*) Same as in *B*, except that cycloheximide (1 μg/ml, ○) or diphtheria toxin (0.1 μM, ●) was added to the cells at 40.8° at 7.5 hr and left in the medium after the downshift to 34° at 8 hr.

The target for α-amanitin (at the concentrations used here) is RNA polymerase II, which is responsible for transcription of mRNA (34). There appears to be some uncertainty about the rate at which it enters the cell and becomes effective, but in our experiments it is clearly active when added at zero time. The dependence of the amount of activity induced on the length of time in the absence of the drug is also not consistent with a very long delay of inhibitor action. The results of the experiment with cordycepin, which prevents polyadenylylation, permit two conclusions: (*i*) the mRNA for Orn decarboxylase is polyadenylylated since addition at zero time suppresses the expression of ODC activity; and (*ii*) the temperature-sensitive block occurs after polyadenylylation. It is not known whether polyadenylylation is the final step prior to export into the cytoplasm. Some recent, very detailed studies on the action of cordycepin by Beach and Ross (36) have emphasized that the specificity of the drug for inhibiting polyadenylylation is not absolute and that RNA synthesis may be affected in general.

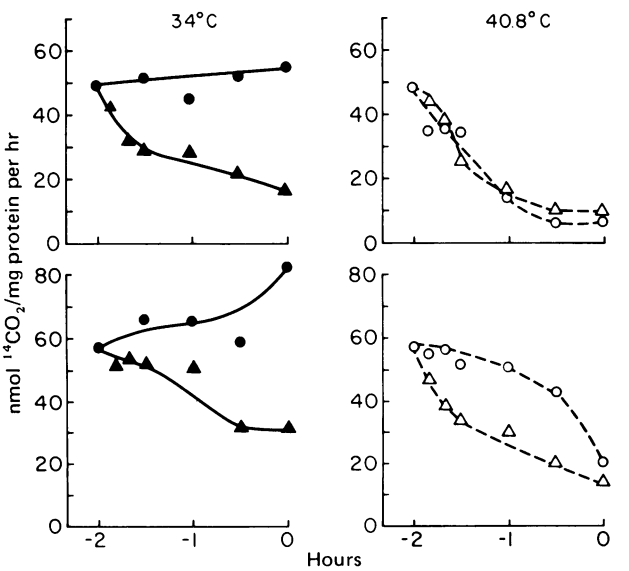

FIG. 5. Orn decarboxylase activity after a temperature shiftup. Enzyme activity was induced in all cells at 34°, after addition of serum to serum-starved cultures. Zero time refers to the time at which the activity is at a maximum in unperturbed cultures. Aliquots of cells were shifted to 40.8° (○, △), and cycloheximide (1 μg/ml) was added (△, ▲) to aliquots at either temperature. The cells were grown as monolayers in erlenmeyer flasks to permit a more rapid equilibration of the temperature, and the 40.8° aliquots were kept in a water bath. At the indicated times samples were removed and frozen, and enzyme activity was determined. (*Upper*) Mutant; (*Lower*) wild type.

Although doubts have occasionally been raised about the interpretation of results obtained with cycloheximide, we find the results to be the same with three different protein synthesis inhibitors, of which diphtheria toxin is not only known to be highly specific (37), but also quite different in regard to its target. Thus, protein synthesis is essential at the permissive temperature for Orn decarboxylase activity to be observed.

To suggest a specific block in translation is perhaps the simplest explanation based on our data, but we are forced to this preliminary conclusion in large part because so little is yet understood of the details of post-transcriptional events. It is possible that the ts defect occurs at such an intermediate stage. The final and unequivocal proof for the accumulation of Orn decarboxylase mRNA in the cytoplasm will have to come from experiments involving translation in a cell-free system of mRNA isolated from the cytoplasm of mutant cells kept at 40.8°. Such an experiment requires a very sensitive means of detection of the enzyme, by its activity, by its immunochemical properties, or by its characteristic migration on a two-dimensional polyacrylamide gel. The latter two properties can only be established with purified enzyme.

It is tempting to speculate on the mechanism of discrimination between different mRNAs, since most proteins are being synthesized *de novo* at 40.8°. The Orn decarboxylase mRNA may reach the cytoplasm in a packaged form that is not immediately capable of interacting with ribosomes. The feasibility of further *in vitro* experiments will depend to a large extent on the relative abundance of this mRNA, which is likely to be rather low.

While the experiments presented here suggest a block in translation of Orn decarboxylase message, there may well be other enzymes or proteins that are not made at 40.8° and that do not have detectable spots in our autoradiogram (Fig. 2). When cell proliferation in wild-type cultures is inhibited by α-methylornithine (38), we found that addition of putrescine to the culture medium could overcome the block. However, at 40.8° the addition of putrescine and the other polyamines to our mutant had no effect on the block in DNA synthesis (unpublished observations). Thus, we believe that our mutant fails to express a series of enzymes, and the common mechanism of control may be at the translational level.

We have already carried out a detailed chracterization of the defect in glycoprotein synthesis in the same mutant (6, 11). It appears that the ts mutation affects the rate of transfer of the oligosaccharide core from the dolichol-pyrophosphoryl-oligosaccharide to the nascent peptides on the rough endoplasmic reticulum, and it is possible that the ts defect is associated with a structural change in intracellular membranes (11). This raises several intriguing possibilities: Orn decarboxylase is a glycoprotein, or intracellular membranes are involved in the control of expression of its activity. These are a few of the possibilities that remain to be tested. A complete understanding of this mutation may reveal fundamental information on the mechanism of control of cell-cycle-specific proteins.

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