

Sequence of Molecular Events Involved in Induction of Allophanate Hydrolase

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Addition of urea to an uninduced culture of *Saccharomyces* at 22 C results in appearance of allophanate hydrolase activity after a lag of 12 min. We have previously demonstrated that both ribonucleic acid (RNA) and protein synthesis are needed for this induction to occur. To elucidate the time intervals occupied by known processes involved in induction, temperature-sensitive mutants defective in messenger RNA transport from nucleus to cytoplasm (*rna1*) and in protein synthesis initiation (*prt1*) were employed along with an RNA polymerase inhibitor in experiments that measure cumulative synthetic capacity to produce allophanate hydrolase. These measurements identify the time within the lag period at which each of the above processes is completed. We observed that RNA synthesis, *rna1* gene product function, and protein synthesis initiation are completed at 1 to 1.5, 4, and 9 to 10 min, respectively.

Precise understanding of the molecular events involved in gene expression begins with identification of the level at which control of expression is exerted, that is, either at transcription or translation. This is followed by a determination of the sequence and time required to complete each of the processes that occur between addition of inducer and appearance of active enzyme. These pieces of information form the framework within which detailed studies of gene regulation must be subsequently carried out.

In previous work (7-9) we have shown that the increase in allophanate hydrolase activity after addition of inducer results from at least two processes. The first is accumulation of an enzyme-forming potential. This is most likely the production of allophanate hydrolase-specific messenger ribonucleic acid (mRNA). Its appearance does not require protein synthesis, but does require the presence of inducer and RNA synthesis. The second process is expression of this accumulated potential or translation of the hydrolase-specific mRNA. This step does not require the presence of inducer or RNA synthesis but does require protein synthesis. Failure of cells to produce allophanate hydrolase when they are growing on readily metabolized nitrogen sources such as glutamine or serine has also been shown (8) to result from transcriptional control of enzyme production. The experiments from which these conclusions were derived depended heavily upon judicious use of transcriptional and translational inhibi-

tors. Difficulties occasionally encountered by others who have performed experiments such as these prompted us to obtain supporting evidence for our conclusions by a different approach. Such an approach is opened by the availability of mutant strains unable to carry out specific steps of transcription and translation. Here we describe experiments, with the temperature-sensitive mutant strains isolated by McLaughlin and Hartwell (2-5), that support our earlier conclusions.

A second objective of the present work concerns the timing of macromolecular synthetic events that occur between addition of inducer and appearance of active enzyme. The times required to complete three such events have been determined and raise a number of questions concerning the primary function of the *rna1* gene product.

MATERIALS AND METHODS

Strains. Our wild-type strain, M25, is a prototrophic, diploid organism prepared as described earlier (12). Temperature-sensitive diploid strains were prepared utilizing standard genetic techniques (10). Haploid mutant strains (α , *ade1*, *ade2*, *gal1*, *his7*, *ura1*, *tyr1*, *lys2*, temperature sensitive) were mated with strain M63-14b (*a*, *leu1*) derived from our wild type, and the resultant diploid strains sporulated. All spores with a genotype of either (*a*, *ade1* or -2, *leu1*, temperature sensitive) or (*a*, *his7*, *ura1*, temperature sensitive) were tested for their ability to produce high levels of allophanate hydrolase upon induction. Diploid strains homozygous for the various temperature-sensitive defects were prepared

using only haploid organisms that produced allophanate hydrolase at high wild-type levels. The temperature-sensitive mutations used here were obtained from McLaughlin and Hartwell and include: *ts-136*, *ts-187*, and *ts-275* which are found in the *rna1*, *prt1*, and *prt3* loci, respectively.

Culture and assay conditions. The medium used throughout these experiments was that of Wickerham (14). Cultures were manipulated and transferred from medium of one temperature to that of another using the procedures of Lawther and Cooper (8). All filtrations using membrane nitrocellulose filters (0.45- μ m pore size; Millipore Corp.) and were completed in less than 15 to 20 s. Since only the membrane filters (Millipore Corp.) containing the harvested cells were transferred to medium at a higher temperature, the time required for temperature equilibration is negligible. In some cases (Fig. 1 and 2) the cells were equilibrated at the higher temperature for an additional 10 min before the experiment was initiated, but such equilibration is not required when cells are transferred from one medium to another in this manner. Enzyme assays were performed essentially as described earlier (1, 12, 13).

RESULTS

Induction of allophanate hydrolase at various temperatures. All of the experiments to be described here, unless noted otherwise, were performed at 22 C, rather than at 30 C as performed earlier (7-9). This change was necessitated by the use of strains carrying temperature-sensitive mutations in various steps of RNA and protein synthesis. These strains grow normally at 22 C but display their mutant phenotype at the nonpermissive temperature of 35 C. A second equally important reason for decreasing the temperature was the technical difficulty encountered in trying to dissect a set of events occurring within a 3- to 5-min time span. By lowering the temperature to 22 C, the lag time between addition of inducer and increases in enzyme level was 12 min, a period that is easily amenable to experimentation (Fig. 1).

Requirement of *rna1*, *prt1*, and *prt3* gene products for allophanate hydrolase production. We have previously shown that production of allophanate hydrolase in response to inducer addition requires both RNA and protein synthesis. Such conclusions were based upon the observations that known inhibitors of RNA and protein synthesis prevented the normally expected increases in enzyme activity. As a means of supporting these results, induction of allophanate hydrolase was monitored in strains defective in various steps of RNA and protein synthesis at 35 C. The three strains we used were derived from those isolated and characterized by McLaughlin, Hartwell, and their

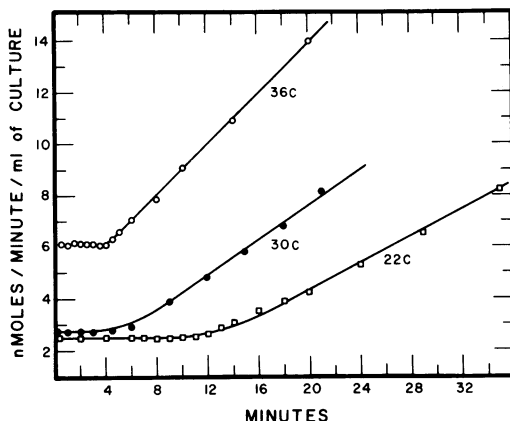


FIG. 1. Time course of allophanate hydrolase induction at various temperatures. Three wild-type cultures (*M25*) of *S. cerevisiae* were grown to cell densities of 30, 30, and 45 Klett units (green no. 54 filter) at temperatures of 22, 30, and 36 C, respectively. One hundred Klett units is approximately equivalent to 3×10^7 cells/ml of culture. At this time each culture was concentrated 10-fold and allowed to equilibrate for 10 min after concentration. After this period, urea (10 mM final concentration) was added to each culture; the time of urea addition is indicated as zero time. Samples were subsequently removed as indicated and assayed for allophanate hydrolase activity.

collaborators and carry mutations *ts-136*, *ts-187*, and *ts-275* in the *rna1*, *prt1*, and *prt3* loci, respectively. Strains carrying a mutation in the *rna1* locus are presumed to be defective in the transport of RNA from the nucleus to the cytosol (2, 5). However, additional evidence will likely be required before this conclusion may be considered unequivocal. The remaining two strains are defective in the initiation of protein synthesis (*prt1*) and the elongation of polypeptide chains (*prt3*) (3, 4). Allophanate hydrolase activity began to increase 12 to 13 min after urea was added to cultures of all of these strains growing at the permissive temperature (Fig. 2). Similar experiments were performed at the nonpermissive temperature and hydrolase activity did not increase, demonstrating that all three of the gene products defective in these strains are required for normal increases in enzyme activity levels.

Since the gene products defective in these strains are all necessary for allophanate hydrolase production, it is reasonable to ask whether or not they are continuously required once induction has been set into progress. For this determination strains containing mutations in the *prt1* and *rna1* loci were induced at 22 C. After enzyme production had reached a constant rate, one-half of the culture was shifted to the nonpermissive temperature while the re-

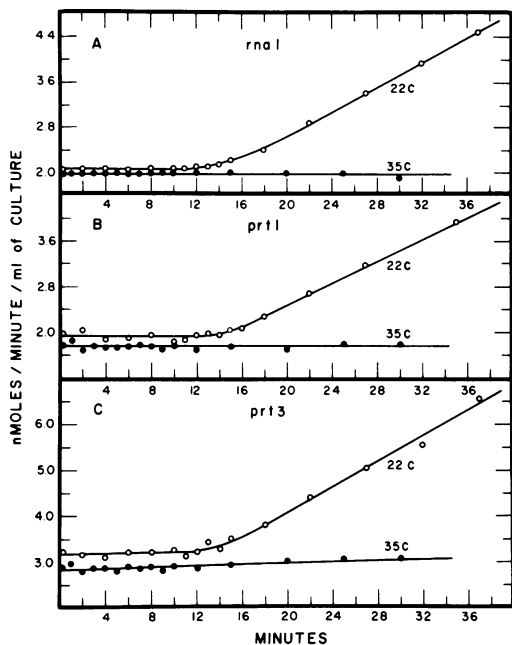


FIG. 2. Induction of temperature-sensitive mutant cultures at permissive and nonpermissive temperatures. Strains carrying mutations in the *rnaI* (A), *prt1* (B), and *prt3* (C) genes were grown to a cell density of approximately 30 Klett units and concentrated 10-fold by centrifugation. Two equivalent samples of each culture were concentrated and equilibrated for 10 min at 22 and 35 C. At zero time, urea (10 mM final concentration) was added to each culture, and samples were removed as described in Fig. 1.

remaining portion was maintained at the original 22 C. Figure 3 demonstrates that increases in allophanate hydrolase activity stopped abruptly after a shift of the strain defective in protein synthesis initiation (*prt1*) to 35 C. The important time period of this experiment was repeated (see inset, Fig. 3) at a higher resolution, and it can be seen that about 3 to 3.5 min elapsed between the temperature shift and cessation of hydrolase production. As indicated in Materials and Methods, no more than 20 s of this time (the duration covered by the heavy black line on the left side of the inset) may be accounted for by operations involved in shifting the temperature from 22 to 35 C. This leaves a period slightly in excess of 3 min within which to inactivate the defective initiation factor and complete (at 35 C) any peptide chains initiated prior to loss of initiation capability. Strains possessing a defective *rnaI* gene product behaved quite differently. Twenty to 25 min elapsed between shifting one-half of the culture to 35 C and loss of its ability to produce allo-

phanate hydrolase (Fig. 4). The half-life of hydrolase-specific synthetic capacity calculated from these data is approximately 4.5 min (see inset, Fig. 4). This is in good agreement with the 3- to 3.5-min value observed when RNA synthesis is terminated by addition of lomofungin or removal of inducer (8, 9). To determine whether or not gross protein synthesis responded in an analogous manner, the experiment described in Fig. 4 was repeated and [3 H]leucine incorporation into hot trichloroacetic acid-precipitable material was monitored in place of allophanate hydrolase activity. Forty-five to 50 min elapsed before [3 H]leucine incorporation ceased (Fig. 5). This yielded a gross protein synthetic capacity half-life of about 15 min, which is significantly greater than the 4- to 5-min value noted above for allophanate hydrolase specifically.

Timing of molecular events in allophanate hydrolase induction. By using a modification of Kepes' methods (6), it is possible to determine when during the 12- to 13-min lag period (Fig. 1 and 2) each of the known synthetic processes involved in allophanate induction occurs. The experimental format has been diagrammed in Fig. 6. At zero time urea was added to one of the mutant cultures growing at 22 C. At various times after this addition, samples of the culture were removed and quickly (20 to 30 s maximum) transferred to the same medium maintained at 35 C. The samples were then permitted to express fully whatever synthetic capacity they might have accumulated at 22 C before being harvested and assayed for enzyme activity (36 min is sufficient time for this to occur). If, for example, a particular function is required 4 min after induction is initiated, transferring samples to the nonpermissive temperature at any time up to 4 min will result in no appearance of enzyme activity. Enzyme fails to appear because at less than 4 min the first hydrolase-specific mRNA has not yet passed the execution point of the required function. If, however, another sample is removed at 6 min, whatever mRNA was produced between 0 and 2 min will have passed the 4-min execution point and can be expressed normally. The remaining mRNA produced between 2 and 6 min will not yet have passed the needed function at 6 min and will fail to be expressed. In this manner it is possible to determine the execution time of each function for which temperature-sensitive mutant strains are available. When this experiment was performed with a strain defective in protein synthesis initiation (*prt1*), 9 to 10 min elapsed before an increase in enzyme activity was observed (Fig. 7B). In a similar experiment

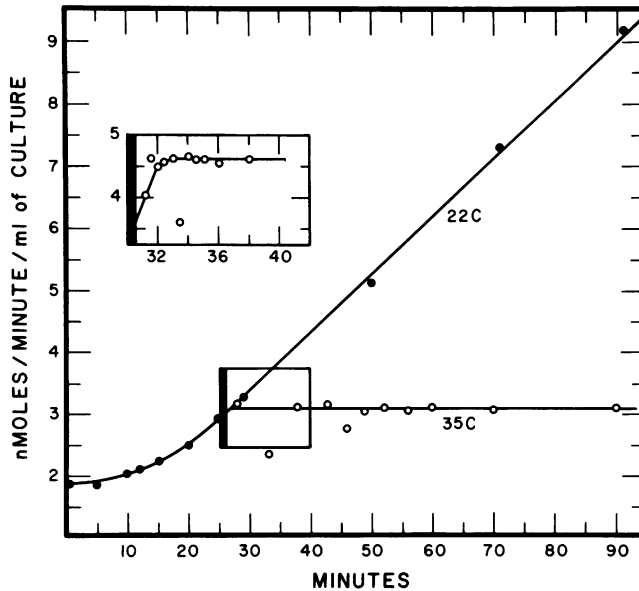


FIG. 3. Loss of ability to produce allophanate hydrolase after a temperature increase to nonpermissive levels. A culture of a strain carrying a temperature-sensitive defect in protein synthesis initiation (*prt1*) was grown at 22 C to a cell density of 35 Klett units in minimal ammonia medium and concentrated 10-fold by centrifugation. At this time (0 min in the figure), urea was added to a final concentration of 10 mM and 1.0-ml samples were removed as indicated. At 25 min a portion of the culture was quickly collected by filtration and resuspended in prewarmed (35 C), pre-aerated medium containing 10 mM urea. Samples (1.0 ml) were removed from both cultures at the indicated times and assayed for allophanate hydrolase activity. Data depicted in the inset were obtained in an identical manner except that samples were removed at shorter time intervals.

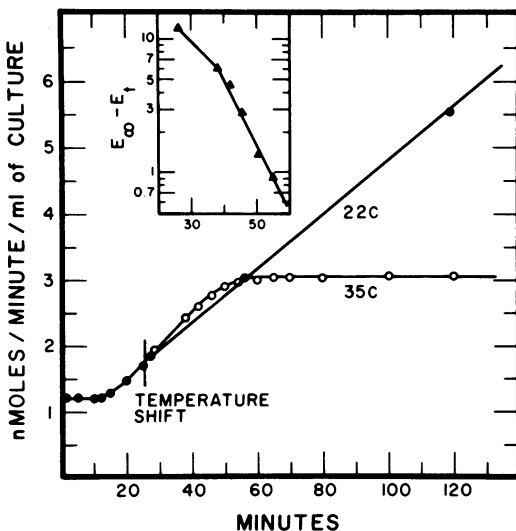


FIG. 4. Loss of ability to produce allophanate hydrolase after a temperature increase to nonpermissive levels. A culture of a strain carrying a temperature-sensitive defect in RNA metabolism (*rna1*) was grown at 22 C to a cell density of approximately 30 Klett units. Subsequent to this point the experimental format was identical to that used in Fig. 3. Data

with a strain carrying a mutation in the *rna1* locus an increase in allophanate hydrolase activity was observed after 4 min, identifying this as the execution time for the *rna1* gene product.

Although temperature-sensitive mutations have been used above, any means of terminating a required synthetic process will work similarly. Therefore, lomofungin can be used to determine the time at which transcription is completed. In this experiment wild-type cells were transferred to flasks containing lomofungin. It should be noted that it is not necessary to raise the temperature of the medium when lomofungin is used. Only about 1 min was required for transcription to be completed (Fig. 7A). Values observed in a number of these experiments ranged from 1 min to slightly less than 1.5 min.

shown in the inset panel were obtained by subtracting the amount of enzyme activity observed at various times after increasing the temperature (O) from the amount observed at 120 min for the culture shifted to 35 C. This difference is denoted as $E_{20} - E_t$. These values were plotted as a function of time on semilogarithmic coordinates.

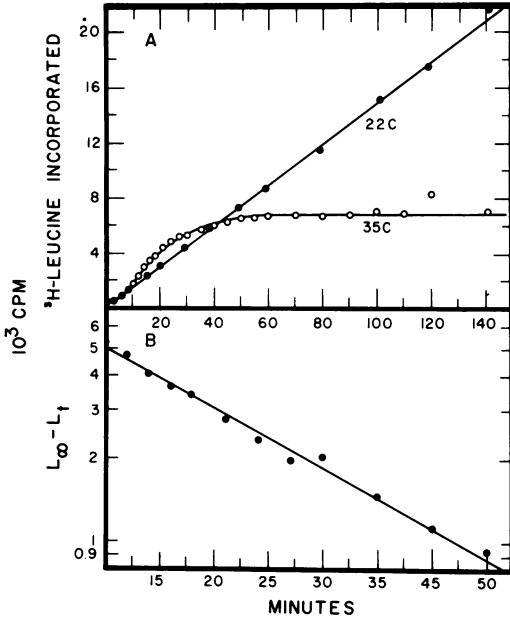


FIG. 5. Loss of ability to produce gross protein after a temperature increase to nonpermissive levels. A culture of a strain carrying a temperature-sensitive defect in RNA metabolism (*rna1*) was grown at 22 C to a cell density of approximately 30 Klett units. At zero time [³H]leucine was added to a final concentration of 5 μg/ml (specific activity, 1 μCi/μg) in place of urea. At the indicated times 0.2-ml samples were removed and added to 5 ml of cold 10% trichloroacetic acid (wt/vol) containing 10 μg of nonradioactive leucine per ml. At 10 min a portion of the culture was quickly collected by filtration and resuspended in prewarmed (35 C), pre-aerated medium containing the same concentration of [³H]leucine as was used in the 22 C medium. Samples were removed from both cultures at the indicated times and processed as described earlier (9). Data shown in (B) were obtained by subtracting the amount of precipitated radioactive leucine observed at various times after increasing the temperature (○) from the amount observed at 140 min for the culture shifted to 35 C. This difference is denoted as L_∞-L_t. These values were plotted as a function of time on semilogarithmic coordinates.

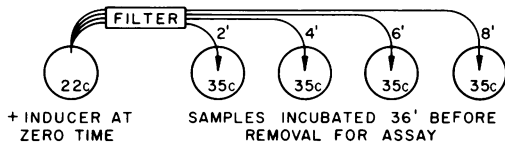


FIG. 6. Experimental format used to measure the cumulative allophanate hydrolase-specific synthetic capacity.

DISCUSSION

We have presented evidence indicating that the *rna1*, *prt1*, and *prt3* gene products are all necessary for production of allophanate hydro-

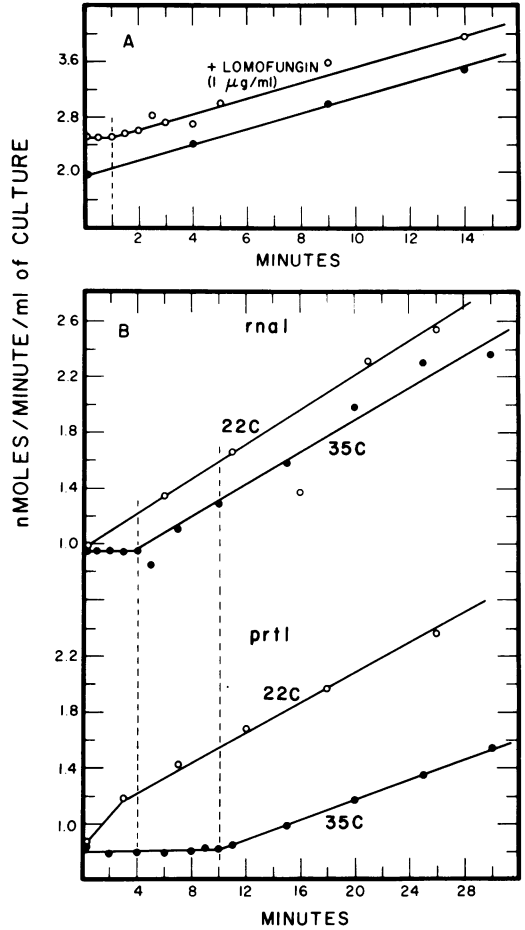


FIG. 7. Cumulative allophanate hydrolase-specific synthetic capacity measured in the presence and absence of various macromolecular synthetic processes. (A) A culture of strain M25 was grown to a cell density of 30 Klett units. Following the experimental format outlined in Fig. 6, the culture was induced with urea and 10-ml samples were removed and added to flasks containing lomofungin (1 μg/ml final concentration, ○). Equivalent samples were transferred to flasks without lomofungin (●). After 35 min of incubation, 5-ml samples were taken and assayed for allophanate hydrolase activity. (B) Cultures of strains carrying a temperature-sensitive defect in RNA metabolism (*rna1*, top) or translation initiation (*prt1*, bottom) were grown to a cell density of approximately 30 Klett units. The cultures were then concentrated 10-fold, and urea was added according to the experimental format described in Fig. 6. One-milliliter samples were collected by filtration as indicated and resuspended in 10 ml of fresh medium without urea at either 22 or 35 C. After 35 min of incubation under these conditions, a 5-ml portion was removed from each sample for assay of allophanate hydrolase.

lase. These findings largely substantiate our earlier conclusions that both RNA and protein synthesis are needed for induction to occur. A temperature-sensitive mutation defective in the actual transcription of mRNA would be required for full support; however, the requirement of a very early function in RNA metabolism is reasonable confirmation of the results we obtained with lomofungin, an RNA synthesis inhibitor.

The results obtained with strains carrying a defective *rna1* gene product not only confirm our earlier ideas concerning the level at which induction is regulated, but also provide a fourth independent means of determining the half-life of allophanate hydrolase-specific synthetic capacity. In present experiments a value of 4 to 4.5 min was obtained. However, included within the 4.5-min period is also the length of time required for the *rna1* gene product to become inactivated at 35 C. If this is taken into account there is good agreement between this result and the 3-min value we reported earlier. The fact that allophanate hydrolase-specific synthetic capacity decays six to seven times faster than the synthetic capacity for gross protein synthesis suggests that *Saccharomyces cerevisiae* likely contains at least two different classes of mRNA, with one class being considerably more stable than the other. The important question raised by such a possibility is whether or not the demonstrated difference in stability of these two classes of synthetic capacities (which are presumably mRNA molecules) reflect two different routes of mRNA metabolism or two structurally different types of mRNA. These possibilities are presently being evaluated.

A major contribution of the present work is identification of the times at which various macromolecular synthetic events are completed during induction of allophanate hydrolase. Transcription is concluded within 1 to 1.5 min after onset of induction (Fig. 8). At 4 min *rna1* gene product is passed, and translation is initiated at 9 to 10 min. Three minutes later active enzyme appears. The most striking aspect of these results is the long period of time between functioning of the *rna1* gene product and trans-

lation initiation. If mRNA transport is assumed to be lost in strains carrying a defective *rna1* gene, one would predict that the execution times of *rna1* and *prt1* gene products would be quite close together. However, present data indicate that they are separated by 4 to 5 min. Interpreted at face value, these observations argue that allophanate hydrolase-specific mRNA spends an improbable 40% of the total induction lag period in the cytoplasm before commitment to translation initiation.

Alternative ways of explaining these results include suggesting that the mutation in the *rna1* locus has affected some step in mRNA processing or an early step in the complex process of transport. In the former view mRNA that is not properly processed cannot be transported. This alternative has been proposed by Shiokawa and Pogo (11). These investigators observed, in agreement with Hartwell and McLaughlin (3), that shifting a strain containing a mutated *rna1* gene to nonpermissive temperatures results in an accumulation of radioactive RNA in the nucleus. They extended the original investigations and reported that lowering the temperature once again to permissive levels results in transport of accumulated RNA out of the nucleus and into the cytoplasm (Fig. 3, reference 11). We repeated their experiment but monitored the ability of cells to produce allophanate hydrolase rather than to incorporate [³H]uridine into poly(A)-containing RNA (Fig. 9A). Unexpectedly, when the temperature was lowered to permissive levels only very small amounts of enzyme could be produced, even though the temperature had been maintained at nonpermissive levels for only 10 min. If the temperature was maintained for 32 min at 35 C before being lowered (this is equivalent to the time reported by Shiokawa and Pogo), no induction at all was observed. To ascertain whether this effect was specific to allophanate hydrolase, we measured the ability of a culture that had been maintained for short times at 35 C to incorporate [³H]leucine into hot trichloroacetic acid-precipitable material after the temperature was decreased to permissive levels. When a culture carrying the *ts-136* lesion was maintained at 35 C for 10 min, its ability to carry out protein synthesis decreased 60 to 70% (Fig. 9B). If the time at 35 C was extended to 30 or 60 min, its ability to carry out protein synthesis was totally lost. These data argue that RNA reported by Shiokawa and Pogo (11) to be transported from the nucleus into the cytoplasm was nonfunctional. However, differences in the media used for their experiments and ours may account for some of the observed disparity.

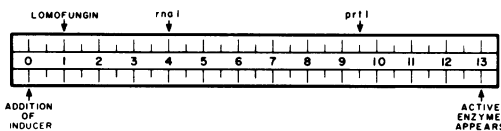


FIG. 8. Sequence and timing of the macromolecular synthetic events involved in the induction of allophanate hydrolase.

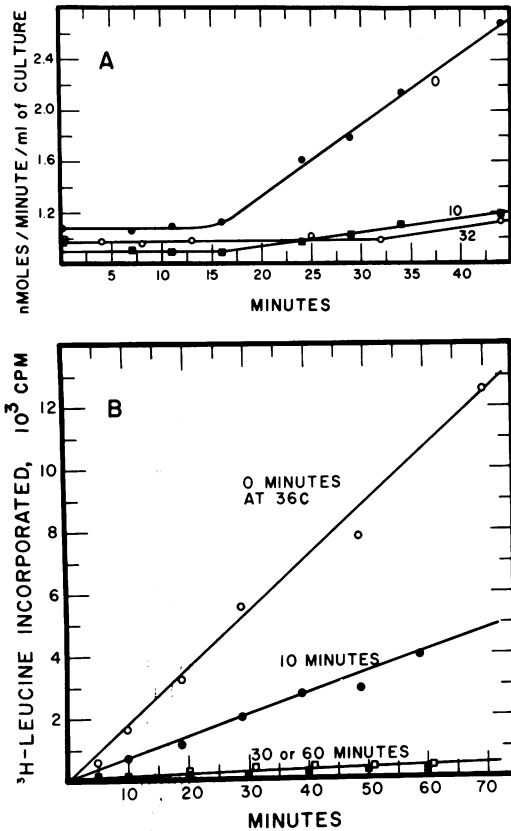


FIG. 9. Synthesis of total protein and allophanate hydrolase in a strain carrying the *ts-136* mutation after incubation at the nonpermissive temperature. (A) A culture carrying a mutation in the *rna1* gene was grown to a cell density of 30 Klett units and concentrated 10-fold. At this time the culture was collected and resuspended in prewarmed (35 C), pre-aerated medium. At 0, 10, and 32 min a portion of the culture was collected by filtration and resuspended in medium containing 10 mM urea at 22 C. Samples from each of the three cultures were removed at the times indicated for assay of allophanate hydrolase activity. (B) A similar experiment was performed. However, the medium (22 C) into which the cultures were resuspended contained 5 μ g of [³H]leucine per ml (specific activity, 1 μ Ci/ μ g). Samples were removed at the times indicated and processed for trichloroacetic acid-precipitable protein as described earlier (9).

A number of significant questions are raised by these results. What is the primary function of the *rna1* gene product? If it does play a role in mRNA transport, why does such a large amount of time elapse between transport of mRNA and initiation of protein synthesis? Is mRNA transport a multistep process involving the sequential operation of a number of gene products? Most importantly, what molecular events occur during the rather long time pe-

riods that precede and follow functioning of the *rna1* gene product?

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