Noncoordinated Transcription in the Absence of Protein Synthesis in Yeast*

Richard W. Shulman, Conjeevaram E. Sripati, and Jonathan R. Warner

In the yeast Saccharomyces cerevisiae, we have carried out a detailed study of the response of transcription to the inhibition of translation. Measurement of the incorporation of labeled bases and nucleosides into the nucleoside triphosphate pools revealed that amino acid deprivation brings about a 10- to 50-fold inhibition of such labeling. Therefore accurate comparisons of RNA synthesis using such precursors are difficult to obtain.

To overcome this problem we have turned to the use of L-[methyl-14C]methionine as a precursor, because the labeling of the S-adenosylmethionine pool is relatively unaffected by the rate of protein synthesis. Using this precursor, we have observed that in the absence of protein synthesis the transcription of ribosomal RNA is reduced by 80%, the transcription of messenger RNA is reduced by about 25%, and the transcription of transfer RNA is reduced by less than 20%. These results are obtained when protein synthesis is inhibited either by deprivation of an amino acid or by the addition of cycloheximide. Ribosomal precursor RNA synthesized in the absence of protein synthesis is fully methylated.

We conclude that the transcription of RNA is the primary target of stringent control. Furthermore, the inhibition of protein synthesis, itself, may be the trigger for this response.

The dependence of RNA synthesis upon simultaneous protein synthesis is a major aspect of intracellular regulation in both prokaryotes and eukaryotes. In bacteria deprivation of an amino acid produces a drastic reduction in the synthetic rates of rRNA (1) and tRNA (2). While the synthetic rate of total mRNA is partially reduced (3), specific mRNAs exhibit highly individual responses (4). Both short pulse (3) and in vitro experiments (4) clearly indicate that the control of RNA synthesis in prokaryotes occurs at the level of transcription.

This set of reactions to amino acid deprivation has been termed the stringent response. The existence of relaxed mutants, relatively unresponsive to amino acid deprivation (5), has led to a thorough study of this phenomenon and the suggestion that the nucleotides ppGpp and pppGpp play a key role (6). An interesting finding is that an intact translational apparatus is necessary to support the stringent response, and that inhibitors of elongation such as chloramphenicol bring about resumption of RNA synthesis (7, 8).

In eukaryotes, little is known about the mechanisms involved in the response of transcription to a block in translation. Deprivation of an amino acid (2, 10), uncharged tRNA (11), blocked initiation (12), and inhibitors of protein synthesis (13-15) inhibit the incorporation of precursors into RNA. Effects on the synthesis and processing of ribosomal precursor RNA have been observed in some instances (10, 16). However, no ppGpp has been reported in yeast although the possibility has been raised for other eukaryotes (see Ref. 17). Also, no relaxed mutants are available.

In order to define more clearly the stringent response in a simple eukaryote, and in anticipation of a search for relaxed mutants, we have undertaken a systematic attempt to determine the role of protein synthesis in the transcription of each of the major classes of RNA in the yeast Saccharomyces cerevisiae.

S. cerevisiae has a number of characteristics which make it a particularly suitable eukaryote for a study of stringency. Auxotrophs are available for rapidly producing a virtual shut off of protein synthesis by amino acid deprivation. RNA can be labeled in very short spans of time with methyl groups from labeled methionine, and the fate of this pulse-labeled RNA can be followed after an effective chase with unlabeled methionine (18).

EXPERIMENTAL PROCEDURES

*This research has been supported by Grants BMS 75 03938 from the National Science Foundation and by Grant NP 72F from the American Cancer Society.
†Supported by United States Public Health Service Grant PO 1CA 13330 from the National Cancer Institute.
§Supported by and on leave from the Centre National de la Recherche Scientifique, France. Present address, Institut de Biologie Physico-chimique, 75005 Paris, France.
¶Faculty Research Awardee of the American Cancer Society.

Yeast Strain, Media, and Cell Growth—Saccharomyces cerevisiae, A364A, a gal-1 ade-2 ura-1 his-7 lys-2 tyr-1 (ATCC 22244), was originally obtained from L. Hartwell, University of Washington, Seattle. Cells were grown overnight at 23° to 1 × 10^7/ml in synthetic medium (SC) (19) containing 14C]cladonine or 14C]uracil. For many of the experiments, the cells were converted to spheroplasts (19, 20), and allowed to recover in SC containing 1 M p-arabitol as osmotic support (SCS) for 1 to 2.5 h before commencing an experiment.

To initiate starvation, two samples were removed from a culture, and the cells collected by centrifugation at room temperature. One aliquot was washed by centrifugation with SCS, and the other aliquot with SCS without tyrosine. The cells were suspended in SCS with or without tyrosine and incubated for 90 to 120 min at 23° (21), after which the cells were labeled as described in individual experi-
ments. Synthesis was stopped by pouring the culture onto frozen 1 M sorbitol.

rRNA and tRNA Labeling, Extraction, and Gel Electrophoresis—The washed spheroplasts were lysed with sodium dodecyl sulfate and RNA extracted with phenol/chloroform/isoamyl alcohol (50/48/2) as previously described (18). The RNA was analyzed by electrophoresis in 2.75% or 10% gels (22) with the sample buffer modified to contain 4 M urea. The gels were sliced and counted as previously described (18).

Determination of mRNA Synthesis—The determination of the synthesis of mRNA was carried out in two ways. (a) Poly(A) containing RNA from cells labeled with [3H]adenine was determined by chromatography on poly (dI) cellulose (23). (b) RNA containing a terminal of the type mG[5']ppp(5')R was determined as described (23) after the removal of tRNA by differential precipitation with 2 M LiCl.

Labeling of Nucleoside Triphosphate Pools—Measurement of the uptake of precursor into the nucleoside triphosphate pools is based on the fact that strain A364A is unable to synthesize either pyrimidines or purines. Therefore, if cells are grown for many generations in the presence of [3H]-labeled adenine or uracil, the uptake of a precursor labeled with [3H] can be measured simply from the [3H]/[14C] ratio of the isolated nucleoside triphosphate. For measurement of the labeling of the ATP and GTP pools, cells were grown for 5 to 8 generations in 0.1 %Ci/0.076 mM of [14C]adenine and pulsed with 30 μCi/0.0015 mM of [3H]adenine. Note that the [3H]adenine caused negligible dilution of the [14C]adenine. At suitable intervals 3-ml aliquots of the culture were chilled on ice and collected by centrifugation. The cells were resuspended in 0.1 ml of H2O to which 0.1 ml of 2 M perchloric acid was immediately added. After transfer to a small plastic tube, the acid-precipitable material was removed by centrifugation for 1 min in a microfuge (Brinkmann model 3200), the supernatant neutralized with KOH, the KC104 removed by centrifugation, and the sample frozen to await analysis. For separation of ATP and GTP, the samples were spotted on a PEI-cellulose thin layer plate (Brinkmann Instruments), which was developed first with H2O, dried, and finally developed with 1.25 M LiCl, 2 N acetic acid (24). ATP and GTP spots were identified by use of standards, cut out, and counted in a toluene base scintillation mixture for determination of the [3H]/[14C] ratio.

Measurement of the labeling of the UTP and CTP pools was essentially the same. Cells were grown in 0.15 μCi/0.092 mm [3H]uracil, and pulsed with 30 μCi/0.0014 mm [3H]uracil, or with 33 μCi/0.0015 mm [3H]uridine. The second development of the PEI-cellulose plate was in 1.25 M LiCl, 1.0 M formic acid (24).

Labeling of S-Adenosylmethionine Pool— Determination of the labeling of the S-adenosylmethionine pool was based on the [3H]/[14C] ratio of AdoMet isolated from cells labeled uniformly with [14C]adenine and briefly with l-[methyl-3H]methionine as previously described (25).

RESULTS

The dependence of RNA synthesis on concomitant protein synthesis in Escherichia coli appears to be due to events occurring at the ribosome (26), events which depend on whether protein synthesis is stopped by deprivation of an amino acid or by the presence of an inhibitor of elongation (27). Attempts to determine whether the situation is the same in eukaryotes have led to conflicting results (28, 29). We have therefore decided to re-examine the relationship between RNA and protein synthesis in yeast with emphasis on two factors often overlooked: (a) the effects of inhibition of protein synthesis on the labeling of precursor pools, and (b) the identification of the types of RNA which are synthesized in the absence of protein synthesis.

The data in Fig. 1, right, show the accumulation of [3H]uridine into macromolecular RNA during the four labeling regimens employed throughout this study. It is apparent that in cells starved for tyrosine, the accumulation of radioactive activity is drastically reduced, while the addition of cycloheximide to starved cells ameliorates this reduction to some extent. Cycloheximide alone causes a 50 to 60% reduction. These effects occur when the rate of protein accumulation has been reduced by 30 to 40% (Fig. 1, left).

Precursor Pools—To determine if some of the results seen in Fig. 1 could be due to pool effects, the uptake of precursors into nucleoside triphosphate pools was determined as described under "Experimental Procedures." The [3H]/[14C] ratios obtained were compared to the control set at 1.0.

| Labeled ad- | ATP  | GTP  | UTP  | GTP  | UTP  | AdoMet |
| position | +Tyrosine | +Tyro- | +Tyro- | +Tyro- | +Tyro- | +Tyro- |
| [3H]Adenine | 1.0 | 0.44 | 0.28 | 0.44 | 0.20 | 0.86 |
| [3H]Uracil | 1.0 | 0.28 | 0.38 | 0.10 | 0.25 | 0.75 |
| [3H]Uridine | 1.0 | 0.44 | 0.46 | 0.10 | 0.46 | 0.86 |
| l-[methyl-3H]- | 1.0 | 0.86 | 0.23 | 0.75 | 0.23 | 0.86 |

* The data for the AdoMet pool is from Ref. 25 and represents a 1-min pulse.
A culture was grown for five generations in complete medium containing 0.03 \(\mu\)Ci/ml of \([\text{H}]\text{uracil}\). The cells were collected by filtration, washed with water, and suspended in medium without tryosine, but containing \([\text{H}]\text{uracil}\) at the same concentration. The culture was divided into four aliquots, two of which received tryosine. After 90 min, two cultures received cycloheximide (100 \(\mu\)g/ml). After 5 min more the cultures containing tryosine received 10 \(\mu\)Ci/ml of \([\text{H}]\text{uracil}\), the ones without tryosine 50 \(\mu\)Ci/ml of \([\text{H}]\text{uracil}\), in anticipation of the poor uptake in the latter case. After 10 and 75 min, samples were harvested from each culture, and the cells washed, disrupted in a Braun homogenizer, and the RNA prepared by phenol-sodium dodecyl sulfate extraction. Samples were chromatographed on poly(dT)-cellulose (23). The data have been corrected for recovery by means of the \(\text{C}^1\text{H}\) radioactivity, and for the different input of \(\text{H}\) on the assumption that because of the cold uracil in the medium, the effective specific activity of the \([\text{H}]\text{uracil}\) in the medium was proportional to the amount of \([\text{H}]\text{uracil}\) added.

### Table II

<table>
<thead>
<tr>
<th>Regimen</th>
<th>10-min label</th>
<th>75-min label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (\text{H})</td>
<td>Bound (\text{H})</td>
</tr>
<tr>
<td>+Tyrosine</td>
<td>25,339</td>
<td>7,398</td>
</tr>
<tr>
<td>+Tyrosine + cycloheximide</td>
<td>14,088</td>
<td>8,247</td>
</tr>
<tr>
<td>-Tyrosine</td>
<td>1,191</td>
<td>620</td>
</tr>
<tr>
<td>-Tyrosine + cycloheximide</td>
<td>1,710</td>
<td>669</td>
</tr>
</tbody>
</table>

**Fig. 2.** Kinetics of labeling of the nucleotide triphosphate pools. Cells were labeled uniformly with a \(\text{C}^1\text{H}\) precursor and pulsed with a \(\text{H}\) precursor as described in the caption to Table I. At intervals after the addition of the \(\text{H}\) precursor, samples were taken and the \(\text{H}/\text{C}\) ratio of purified nucleoside triphosphates was determined as described under "Experimental Procedures." A, precursor, \([\text{H}]\text{adenine}; \text{O}, \text{ATP}; \text{O}, \text{GTP}; \text{B}, \text{precursor}, \([\text{H}]\text{uracil}; \text{O}, \text{UTP}; \text{O}, \text{CTP}.\)

As expected, it inhibits labeling only partially in complete medium. These data lead us to suggest that amino acid starvation itself, rather than the rate of protein synthesis, plays a major role in regulating the uptake of bases and nucleosides.

In comparing the data of Fig. 1 and Table I it becomes apparent that much of the stringency observed in measuring the incorporation of labeled nucleotides into RNA is due to an effect on pool equilibration rather than on transcription.

**Synthesis of Poly(A) Containing RNA in Absence of Protein Synthesis**—It has recently become possible to identify most mRNA by its 3'-poly (A) terminus (30). In yeast more than 80% of all poly(A) containing RNA is in the cytoplasm.2 The data in Table II show that the proportion of radioactivity in poly(A) containing RNA is substantially greater in cells unable to synthesize protein than in growing cells. This result suggests that mRNA synthesis is less susceptible to inhibition under these conditions than is RNA synthesis.

**Use of Methyl Labeling to Determine RNA Synthesis**—In previous work, we observed that the labeling of the AdoMet pool with L-[methyl-\(\text{H}\)]methionine occurs very rapidly, and is affected only slightly by the inhibition of protein synthesis within the cell (25) (see Table I). We have therefore used methyl labeling of RNA to determine more precisely the relative rate of synthesis of the different classes of RNA in cells under various regimens.

**Synthesis of mRNA in Absence of Protein Synthesis**—In all eukaryotes, molecules of mRNA in yeast have an unusual structure at the 5' terminus: m7G(5')ppp(5')R, where R is either adenosine or guanosine in a ratio of 3 to 2 (23). The mRNA has no other methyl group than the 5'-terminal methyl exchange in the cytoplasm.3 Total RNA was isolated from cells labeled for 7 min with L-[methyl-\(\text{H}\)]methionine. The 5' ends were isolated as described in Ref. 23. Table IV shows the incorporation of \(\text{H}\) into 5'-terminal structures by spheroplasts cultured under the various regimens. Clearly the synthesis of mRNA is diminished only moderately when protein synthesis is interrupted either by addition of cycloheximide or by starvation for tyrosine. Correction for the AdoMet pools was not carried out in this case, since by 7 min the pools

<table>
<thead>
<tr>
<th>Regimen</th>
<th>(\text{H}) in 45 min chase</th>
<th>(\text{H}) corrected for AdoMet pool specific activity</th>
<th>Relative RNA synthesis (\text{cpm})</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Tyrosine</td>
<td>5077</td>
<td>5077</td>
<td>1.0</td>
</tr>
<tr>
<td>+Tyrosine + cycloheximide</td>
<td>4265</td>
<td>4959</td>
<td>0.98</td>
</tr>
<tr>
<td>-Tyrosine</td>
<td>2983</td>
<td>3990</td>
<td>0.79</td>
</tr>
<tr>
<td>Tyrosine + cycloheximide</td>
<td>4364</td>
<td>5974</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Corresponding to frozen 1 M sorbitol, the subsequent extraction and processing cultures were labeled for 7 min with 63 pCi/ml of L-[methyl-3H]methionine were identical with those described in Fig. 3. The pulse was terminated by pouring the cultures onto frozen 1 M sorbitol, the subsequent extraction and processing was as described under "Experimental Procedures" and Ref. 18.

The interruption of mRNA synthesis.

The extent of processing of that precursor rRNA which is synthesized during the pulse can be seen in the analysis of the 10-min chase (Fig. 3, B, D, F, and H). In the control, nearly all of the precursor rRNA has been processed to the mature 25 S and 18 S forms and no 35 S remains. Under conditions of inhibition of protein synthesis very little pulse-labeled RNA has been processed to the mature forms. The RNA is seen almost exclusively as the 35 S transcript. Thus, processing of ribosomal RNA is virtually eliminated in the absence of protein synthesis.

Quantitative Analysis of rRNA Transcription

We would like to make a quantitative analysis of rRNA transcription under conditions described in Fig. 3. However such an analysis depends on the assumption that ribosomal precursor RNA is fully methylated in cells starved for an amino acid. Since processing of ribosomal precursor RNA is inhibited in such cells, it seemed possible that methylation could also be affected. We were able to rule out such a complication by means of the experiment described in Fig. 4. This experiment is based on the fact that more than 95% of the methylation of HeLa ribosomal precursor RNA occurs on the 2'-OH of the ribose (33), and a similar situation appears to exist in yeast (34). Since the presence of a 2'-O-methyl renders the adjacent phosphodiester bond resistant to alkaline hydrolysis, the level of such alkali-resistant dinucleotides is a measure of the degree of methylation of the RNA.

In Fig. 4 it is apparent that the 35 S RNA synthesized in cells starved for tyrosine has both di- and trinucleotides resistant to alkaline hydrolysis. Table V shows that the level of ribose methylation is essentially unaffected by the lack of protein synthesis.

Therefore, it is now possible to determine the relative rates of rRNA transcription under various conditions by measuring the radioactivity incorporated into ribosomal precursor RNA during a short pulse of L-[methyl-3H]methionine, after correction for the specific activity of the AdoMet pool (Table I). Such a determination for the experiment of Fig. 5 is summarized in Table VI.

Clearly, interference with protein synthesis either by starvation for an amino acid or by addition of cycloheximide results in a marked reduction in the rate of rRNA transcription. The addition of cycloheximide to a culture starved for an amino acid does not bring about a resumption of rRNA synthesis.

While the finite size of the AdoMet pool hampers precise analysis of the fate of the pulse-labeled RNA, the data in Table

### Table IV

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Specific activity</th>
<th>Relative mRNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Tyrosine</td>
<td>3126</td>
<td>1.0</td>
</tr>
<tr>
<td>+Tyrosine + cycloheximide</td>
<td>2069</td>
<td>0.66</td>
</tr>
<tr>
<td>-Tyrosine</td>
<td>2358</td>
<td>0.75</td>
</tr>
<tr>
<td>-Tyrosine + cycloheximide</td>
<td>2234</td>
<td>0.74</td>
</tr>
</tbody>
</table>

**Fig. 3.** Analysis of methyl-labeled ribosomal RNA. Cells were grown overnight in SC containing 0.05 μCi/ml of [3H]methionine. The cells were converted to spheroplasts and allowed to recover in complete SC containing [3H]methionine at the same concentration. At the end of 60 min the culture was divided into four aliquots and the spheroplasts washed once with the medium in which subsequently they were cultured. Two aliquots contained SCS, and two aliquots, SCS-Tyr, all with [14C]uracil at the same concentration. After 90 min two aliquots received 10 μg/ml of cycloheximide. After 60 min each aliquot was labeled with 100 μCi/ml of L-[methyl-3H]methionine. A sample was taken at the end of 1 min and 500 μg/ml of unlabeled methionine added to the remainder. Samples were taken 1 min and 10 min later. The spheroplasts were collected by centrifugation, lysed, and their RNA extracted and subjected to electrophoresis in 2.75% acrylamide gels as described under "Experimental Procedures." The data have been normalized for the 10C long label in the gels. A and B, tyrosine; C and D, +tyrosine + cycloheximide; E and F, -tyrosine - cycloheximide; G and H, -tyrosine + cycloheximide. A, C, E, and G contain the 1-min pulse (●) and 1-min chase (○). B, D, F, and H contain the 10-min chase (●—○).
nucleoside triphosphate pools, especially the derivative ones, GTP and CTP (Table I). Cycloheximide has much less effect, bition of the uptake of adenine, uracil, or uridine into the tion. options of ribosomal precursor RNA, but not its rapid degradation. VI indicate little or no loss of labeled RNA in the cultures where protein synthesis has been inhibited. Thus, the absence of protein synthesis brings about the inhibition of transcription of ribosomal precursor RNA, but not its rapid degradation.

**DISCUSSION**

**Pools**—Starvation for an amino acid causes a dramatic inhibition of the uptake of adenine, uracil, or uridine into the nucleoside triphosphate pools, especially the derivative ones, GTP and CTP (Table I). Cycloheximide has much less effect, and even ameliorates to some degree the effects of amino acid starvation. These results lead us to speculate that the uptake of bases and nucleosides is controlled at two levels: (a) Inhibition of translation leads to a moderate decrease of uptake into nucleoside triphosphates. (b) Deprivation of the amino acid, tyrosine, leads to nearly total inhibition of such uptake. If this effect is mediated by an unchanged tyrosyl-tRNA, addition of cycloheximide could lead to a “trickling-charging” of the tRNA, partially reversing the inhibition of nucleotide labeling.

**Accumulation**—The regulation of RNA synthesis is often studied with experiments such as that in Fig. 1. It is clear that these can be very misleading in the absence of a detailed kinetic analysis of the labeling of the nucleotide pools (Table I). Such studies are subject to additional error because they do not distinguish between the synthesis of stable RNA and of unstable RNA. The latter makes up at least 30% of the initial rRNA, and perhaps as much as 75% in cultured mammalian cells (35). The turnover of such RNA and the increasing specific activity of the nucleotide pools can balance each other to provide a neat straight line which is an utterly misleading picture of transcription.

**Transcription**—The data presented in Tables III, IV, and VI indicate that the interruption of protein synthesis for a period of 2.5 h has little if any effect on the synthesis of tRNA, a marginal effect on the synthesis of mRNA, and a profound effect on the synthesis of rRNA. We conclude that the regulation of the transcription of the three types of RNA is not coordinate. Whether this is due to the use of different polymerases or to the use of different signals remains to be seen.

In normal cells transcription is divided roughly as follows: rRNA, 60%; tRNA, 10%; mRNA, 30%. Therefore the data suggest that, in the complete absence of translation, cells can continue transcription at nearly 50% of the control rate.

In our experiments, cycloheximide, when added to cells starved for an amino acid, had no effect at the level of transcription. We suggest that much of the apparent “phenotypic reversion” observed by others (21, 29) may be due to effects on the labeling of nucleotide pools. The response of yeast to cycloheximide is quite different than the response of bacteria to chloramphenicol. In yeast, cycloheximide inhibits rRNA synthesis in the presence or absence of an essential amino
Noncoordinate Transcription in Yeast

acid, while in bacteria chloramphenicol permits rRNA synthe-
sis in the presence or absence of amino acids (36, 37). Tempera-
ture-sensitive mutants of yeast, defective in the initiation of
polypeptide synthesis (11, 12), also fail to accumulate RNA.
The uniform response of yeast to various modes of protein
synthesis inhibition suggests that insufficient protein synthe-
sis itself may trigger the inhibition of rRNA transcription
which is characteristic of the stringent response.

In certain temperature-sensitive mutants of yeast, the syn-
thesis of ribosomal proteins is specifically interrupted (38, 39).
Since, in such mutants, the transcription of ribosomal RNA is
relatively unaffected, the stringent response we have ob-
served in Fig. 3 is not due to a specific lack of ribosomal
proteins.

The partial inhibition of synthesis of mRNA is of interest in
view of the observations that in Escherichia coli starved for an
amino acid the synthesis of some mRNA molecules is en-
chanced while the synthesis of others is inhibited (4). Cell-free
translation experiments to test such a possibility in yeast are
in progress.

REFERENCES
2. Ikemura, T., and Dahlberg, J. E. (1973) J. Biol. Chem. 248, 5033-
5041
420-429
Natl. Acad. Sci. U. S. A. 70, 2881-2885
5. Block, R., and Haseltine, W. A. (1974) in Ribosomes (Nomura,
Spring Harbor Laboratory, New York
Harbor Laboratory, New York
Acta 53, 79-84
Acta 53, 96-110
68, 118-129
Bacteriol. 100, 759-758
Sci. U. S. A. 62, 468-474
Biol. 36, 91-109
41, 177-187
1697-1705
22. Peacock, A. C., and Dingman, C. W. (1967) Biochemistry 6, 1818-
1827
Chem. 251, 2988-2994
Biochem. 13, 211-222
Bacteriol. 125, 887-891
Biol. 11, 131-228
181, 1215-1221
32. Vaughan, M. H., Sveito, R., Warner, J. R., and Darnell, J. E.
Biophys. Acta 185, 370-380
35. Sveito, R., Vaughan, M. H., Warner, J. R., and Darnell, J. E.
Mol. Gen. Genet. 109, 42-56
U. S. A. 73, 1547-1551

* Unpublished observations.