The Control of Ribonucleic Acid Synthesis in Escherichia coli

IV. RELEVANCE OF UNUSUAL PHOSPHORYLATED COMPOUNDS FROM AMINO ACID-STARVED STRINGENT STRAINS

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SUMMARY

Stringent strains of Escherichia coli are shown to produce one, and usually two, 32P-labeled compounds specifically in response to amino acid starvation. This pattern of labeling is not observed in three derived relaxed strains. Comparison of properties of the system regulating the synthesis of these compounds with those of the amino acid control of RNA biosynthesis suggests that the unusual compounds participate in an early step in the mechanism which leads to the slowing of RNA biosynthesis during amino acid starvation of stringent strains.

The limitation of amino acid availability for protein synthesis in "stringent" bacteria (phenotypically RCst) limits the rate of RNA synthesis (see reviews of Neidhardt (1, 2), Maaløe and Kjeldgaard (3), Edlin and Broda (4)). Much less stringent is the amino acid dependence of RNA synthesis in "relaxed" mutants (RCrel) which were first described by Borek, Roekenbach, and Ryan in 1956 (5) and later isolated in several laboratories. This phenotypic difference has in most, but not all, cases been ascribed to a mutation in a single genetic locus, the RC gene (6-8). Neither the mechanism which leads to the stringent response nor the means by which mutations can alter the amino acid control of RNA synthesis is fully understood.

Experiments have previously been described in which supplementation of plasmolyzed cell preparations with the four ribonucleoside triphosphate substrates of RNA polymerase abolished the stringent response, whereas the substitution of labeled UMP or uracil for UTP resulted in amino acid-dependent incorporation of label into RNA (9). The suggestion that amino acid starvation is imposed, suggest that the appearance of the unusual phosphorylated compounds precedes the slowing of uracil incorporation into RNA when amino acid starvation is imposed, suggest that the appearance of these compounds is related to an early step in the mechanism leading to the stringent response to amino acid starvation.

MATERIALS AND METHODS

Bacterial Strains—Several of the E. coli strains utilized here have been described previously (10). B333 is a derivative of B3 and required leucine, methionine, proline, and thymine. It is RCst. Strains NP 2 and NP 29, obtained from Dr. F. C. Neidhardt, are described in the literature as KB and 10, respectively (13). NP 29 is a temperature-sensitive (40°) valyl-tRNA synthetase mutant derived from NP 2. Both strains are prototrophic and phenotypically stringent. The permissive temperature employed was 30°.

Three pairs of strains were employed which are closely related except for differences in the allelic state of the RC gene. EA 1 (RCst) and EA 2 (RCrel) are K-12 strains; EA 1 is of 58-161 lineage. Both members of this pair require methionine and EA 1 also requires biotin. The isogenic 15 TAU RCst and 15 TAU RCrel pair of strains were obtained from Dr. D. Ezekiel and require thymine, arginine, and uracil. CP 78 (RCst) and 79 (RCrel) were obtained from Dr. C. Edlin and are also isogenic except at the RC locus; both require arginine, leucine, histidine, threonine, and thiamine.

Media and Growth Conditions—A Tris-glucose minimal medium (9) was routinely employed. Amino acids were present when required at 20 µg per ml. Nucleoside requirements were present at 10 µg per ml, glucose at 0.2%, and KH₂PO₄ at 0.002 M. The isotope labeling medium consisted of 10 to 50 µCi per ml of ³²P; added to Tris-glucose media containing 2 × 10⁻⁴ M KH₂PO₄ and 2 × 10⁻³ KCl. Bacterial cultures were maintained at 37°.
Formic Acid Extraction and Chromatography—Acid extraction of labeled bacterial cultures was accomplished by diluting a measured aliquot of cells (usually 50 μl) with an equal volume of 2 M formic acid. After incubating in an ice bath for at least 15 min, the suspension was transferred to a Beckman microfuge tube, and the cells were sedimented for 1 min. The cell-free supernatants were spotted directly on polyethyleneimine-cellulose chromatograms or were frozen and chromatographed later. The 32P-labeled compounds of interest are stable in the formic acid extracts for at least 2 weeks when frozen; the extracts were rarely stored for longer periods because of isotope decay. The two MS compounds are not extractable with 5% trichloracetic acid or 6% perchloric acid, but can be recovered by extracting cells with ethanol or distilled water.

Polyethyleneimine-cellulose thin layer sheets were obtained commercially (Brinkmann Instruments) and prior to use were soaked in distilled water for at least 20 min, then dried at room temperature with a fan. Although the MS compounds may be detected by two-dimensional thin layer chromatography methods employed earlier (12), a simpler procedure (14) is used here which gives quantitative resolution of these compounds after development of the chromatograms in only one dimension. This consists of ascending development with 1.5 M KH₂PO₄ (pH 3.4) to 17 cm above the origin (the top of the plate) in closed chambers at room temperature. Fig. 1 shows that MS compounds are well resolved from other phosphorylated compounds present in acid extracts of 32P-labeled E. coli. The MS I and MS II compounds migrate about 4 cm and 2 cm from the origin, respectively, whereas GTP migrates about 7 cm from the origin (see Fig. 1). The origin contains a variable amount of radioactive material from formic acid extracts. Quantitation of radioactivity is by radioautographic localization followed by counting the appropriate cutouts in scintillation medium (Liquifluor, New England Nuclear Corporation) (15). Absolute incorporation of 32P was calculated from counts obtained with aliquots (5 μl) of reaction mixtures spotted directly on polyethyleneimine-cellulose cutouts, dried, and counted.

Materials and Isotope Techniques—Thiazole alanine was obtained from Cyclo Chemical Corporation (lot F-1771) and venom phosphodiesterase from Worthington. For estimation of the 32P in the α and β + γ positions in thin layer chromatography-purified GTP, the enzyme was employed at O.D.₃₆₅ = 0.76 in reaction mixtures containing 0.1 M Tris-HCl (pH 8.9), 0.001 M MgCl₂, and thin layer chromatography-purified GTP. The course of the hydrolysis reaction was measured by chromatographing aliquots of the mixture and hydrolysis was judged complete when GTP completely disappeared and GMP ceased to be produced (about 5 min at 37°). Later aliquots were used for quantitation of radioactivity appearing in GMP and pyrophosphate. During 30-min incubation no appreciable inorganic phosphate appeared, indicating little pyrophosphatase activity during this period. GTP was isolated by preparative chromatography with 1.5 M KH₂PO₄, eluted from the appropriate region of the chromatogram with 4 M LiCl (eluante pH 3.4), and purified free of non-nucleotide phosphates by the Norit-Celite adsorption and elution conditions described by Roblin (16). This eluate was dried by flash evaporation at room temperature and resuspended in a small volume of distilled water resulting in activities of about 5 × 10⁶ cpm/5 μl.

Silicate-free preparations of 32P-labeled (carrier-free) orthophosphoric acid were obtained from Tracer Laboratories. Chromatographic analysis for the presence of polymerized phosphate revealed only trace amounts of origin-bound radioactivity and the remainder of the radioactivity migrated with orthophosphate. Accordingly, the preparations were not boiled in acid prior to use. Tritiated uracil (New England Nuclear Corporation) used for pulse labeling experiments was present at 5 μCi/0.56 mmole/50 μl of cells. The addition of the cells to tubes prewarmed to 37° (to evaporate the 5 μl of uracil solution added as well as to maintain a constant pulse temperature) was made with 50-μl Eppendorf automatic pipette (Brinkmann Instruments). The pulse was terminated 20 sec later with 1 ml of ice cold 5% trichloracetic acid. After incubation in ice for at least 30 min, the samples were filtered through Millipore (HA) filters, washed with 5% trichloracetic acid, and counted in Bray’s liquid scintillation medium (17).

Colorimetric Estimation of RNA and Protein—Aliquots of cultures were diluted with 0.1 volume of 50% (w/v) trichloracetic acid and placed in ice for 30 min. The cells were then sedimented and washed twice with 2 volumes of 5% trichloracetic acid. The resulting cell pellets were assayed for RNA by the orcinol procedure (18) and for protein by the phenol procedure (19) using as standards yeast RNA or bovine serum albumin.

RESULTS

Effect of RC Alleles—It has been previously reported that the majority of phosphorylated compounds extractable from stringent strains with formic acid are more heavily labeled with 32P in the presence of Casamino acids than without supplementation (12). In contrast to this pattern of labeling, two compounds (MS I and MS II) were detected when cultures of stringent strains were amino acid-starved. The experiments that follow are designed to ascertain the relevance of amino acid control of MS compounds to the amino acid control of RNA biosynthesis.

In order to determine in more detail whether the appearance of MS compounds is affected by the allelic state of the RC gene, assays for MS were performed on three stringent-relaxed pairs of otherwise closely related E. coli strains. To impose a specific amino acid starvation, the cells were grown in minimal media supplemented only with required amino acids. They were then filtered, washed, and resuspended in media containing 32P, which was either lacking a required amino acid as specified in Table I or supplemented with all of the required amino acids. Formic acid extraction was performed after 30-min incubation at 37° and the cell-free supernatant was chromatographed as described under “Materials and Methods.” Radioactivity appearing in both compounds during this incubation reaches a plateau level after 15 to 20 min and this level persists for at least an additional hour of starvation. It is likely that MS is uniformly labeled with 32P after 30 min of incubation since GTP (isolated from B333 and purified as described under “Materials and Methods”) labeled during this period and hydrolyzed by venom phosphodiesterase gives a ratio of 32P in the α position to that in the β + γ positions of 1:2.06.

Fig. 1 shows the effects of incubating cultures with 32P, in the presence or absence of amino acids. For each strain the effects of amino acid starvation were determined in a paired experiment with a constant specific activity of 32P. Among the different strains the range of specific activities was 5 × 10⁴ to 9 × 10⁵ cpm per mmole of phosphate per 5 μl.
TABLE I

Quantitative results obtained for MS activity in survey of cell strains under conditions of amino acid starvation (−) or supplementation (+).

The appropriate areas of the chromatogram giving rise to the radioautogram shown in Fig. 1 were counted for radioactivity as described under “Materials and Methods” and the resultant activities tabulated as millimicromoles of phosphate per optical density unit of bacteria. Phosphate activities were 5 to 9 × 10⁴ cpm per nanomole of phosphate per 5-μl culture. Required amino acids other than the one indicated were present in the media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>MS I</th>
<th>MS II</th>
</tr>
</thead>
<tbody>
<tr>
<td>B333</td>
<td>- Leucine</td>
<td>2.66</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>+ Leucine</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>CP 78</td>
<td>- Leucine</td>
<td>1.94</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>+ Leucine</td>
<td>0.12</td>
<td>0.03</td>
</tr>
<tr>
<td>CP 79</td>
<td>- Leucine</td>
<td>0.05</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td></td>
<td>+ Leucine</td>
<td>0.04</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>15 TAU RC&lt;sup&gt;sr&lt;/sup&gt;</td>
<td>- Arginine</td>
<td>2.13</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>+ Arginine</td>
<td>0.38</td>
<td>0.05</td>
</tr>
<tr>
<td>15 TAU RC&lt;sup&gt;rel&lt;/sup&gt;</td>
<td>- Arginine</td>
<td>0.43</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>+ Arginine</td>
<td>0.32</td>
<td>0.04</td>
</tr>
<tr>
<td>EA 1</td>
<td>- Methionine</td>
<td>2.22</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>+ Methionine</td>
<td>0.43</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>EA 2</td>
<td>- Methionine</td>
<td>0.06</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td></td>
<td>+ Methionine</td>
<td>0.09</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>EA 1</td>
<td>+ 18 amino acids</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
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</table>

With strain B333 it may be noted that polyethyleneimine-cellulose chromatography in 1.5 m KH₂PO₄ resolves MS I and MS II (for the faster and slower moving compounds, respectively) from other phosphorylated compounds in acid extracts. Neither MS I nor MS II appears heavily labeled in the culture supplemented with leucine, but both are heavily labeled in the culture which is leucine-starved. When formic acid was added to the cells prior to the addition of isotope there was no radioactivity in the MS region of the chromatogram.

The stringent strain CP 78 also produces MS I as well as MS II when leucine starvation is imposed, but not when the medium is supplemented with this required amino acid. The isogenic relaxed strain CP 79, does not yield either MS compound, with or without starvation for leucine. Assays with the 15 TAU pair of stringent-relaxed strain confirms this effect of the rel allele on the appearance of MS I and MS II.

In the case of the EA 1-EA 2 pair of strains, the stringent (EA 1) produces an increase in the activity of ³²P appearing in MS I after methionine starvation. EA I is unlike the other stringent strains in that it does not produce MS II after methionine starvation. Since the other methionine-requiring stringent strain (B333) does produce MS II after methionine starvation (see below), this atypical behavior of EA 1 is not due to methionine starvation but appears to be specific to strain EA 1. The relaxed member of this pair of strains (EA 2) produces no MS after amino acid starvation and thus behaves like the other two relaxed strains. Close examination of the radioautogram from the culture of the stringent strain EA 1 supplemented with methionine reveals trace amounts of MS I. With the other strains growing...
cells in media lacking any two, or all three, required amino acids. They were then harvested, washed, and incubated as in Fig. 1 at $O.D._{760} = 0.25$. $^{3}P$-specific activities were $1.6 \times 10^{6}$ cpm per mmole in 5 μl (B333) and $7 \times 10^{6}$ cpm per mmole in 5 μl (CP 78). Valine and thiazole alanine additions were 200 μg per ml and 50 μg per ml, respectively.

Amino acid specificity

Table II

<table>
<thead>
<tr>
<th>Condition</th>
<th>MS I</th>
<th>MS II</th>
</tr>
</thead>
<tbody>
<tr>
<td>B333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete (+ met, leu, pro)</td>
<td>≤0.1</td>
<td>≤0.1</td>
</tr>
<tr>
<td>Met</td>
<td>2.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Leu</td>
<td>2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Pro</td>
<td>2.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Met, leu</td>
<td>1.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Met, pro</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Pro, leu</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Met, leu, pro</td>
<td>2.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Complete + thiazole alanine</td>
<td>3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Complete + val</td>
<td>≤0.1</td>
<td>≤0.1</td>
</tr>
<tr>
<td>CP 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete (+ leu, thre, his, arg)</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Leu</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Thre</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>His</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Arg</td>
<td>2.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Leu, thre, his, arg</td>
<td>1.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Complete + val</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Complete + thiazole alanine</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Stringent strains B333 and CP 78 were grown to $O.D._{760} = 0.4$ in Tris-glucose minimal medium supplemented with required amino acids. They were then harvested, washed, and incubated as in Fig. 1 at $O.D._{760} = 0.25$. $^{3}P$-specific activities were $1.6 \times 10^{6}$ cpm per mmole in 5 μl (B333) and $7 \times 10^{6}$ cpm per mmole in 5 μl (CP 78). Valine and thiazole alanine additions were 200 μg per ml and 50 μg per ml, respectively.

Starvation of CP 78 for any of its four amino acid requirements also results in the appearance of MS I and MS II. Thiazole alanine addition to this histidine auxotroph does not lead to the appearance of MS compounds since histidine is present in the media, but histidine starvation by filtration and resuspension in histidine-free media does. The production of MS occasioned by thiazole alanine addition is therefore attributable to histidine starvation.

Table II also shows that valine addition results in the appearance of label in MS in the K strain (CP 78) but not in the B strain (B333). This effect is apparently caused by false feedback inhibitor activity of valine on the isoleucine biosynthetic pathway also occurring in K, but not in B, strains of E. coli (23). MS compounds are not detectable 10 min after isoleucine (100 μg per ml) has been added to the valine-inhibited culture; this also suggests that the appearance of MS in CP 78 is attributable to isoleucine starvation imposed by the addition of valine. Altogether it is very likely that the appearance of MS is not specific for the particular amino acid for which starvation is imposed. MS appearance shows the same lack of specificity toward specific amino acids as does the amino acid RNA control mechanism.

Effects of Impairment of Aminocar-yl-tRNA Synthetase Activity—Since it is likely that MS appears in cultures that have been starved for any amino acid, and since amino acid control of RNA biosynthesis shows a similar pattern, the question arises whether this effect on MS appearance is mediated by the free amino acids themselves or by the lack of a full complement of active aminocar-yl-tRNA synthetase reactions. The latter has been clearly implicated in the amino acid control of RNA synthesis with mutants having altered p-fluorophenylalanyl-tRNA synthetase activities (24, 25), as well as with a mutant carrying a temperature-sensitive valyl-tRNA synthetase (13). The temperature-sensitive strain (NP 29) as well as its temperature-insensitive parent (NP 2) were assayed for the presence of MS at permissive (30°) and restrictive (40°) temperatures. Fig. 2 shows the results, which indicate that impairment of valyl-tRNA synthetase activity leads to the rapid appearance of MS I and MS II even though 0.2% Casamino acids are present in the culture medium. The temperature-insensitive strain NP 2 does not produce MS at 40° under the same conditions. However, if aeration is interrupted at high cell densities, cultures of the parental strain produce both MS compounds, showing that NP 2 has the capacity to produce MS. Cell growth and RNA synthesis (trichloracetic acid-precipitable $^{3}P$) were measured for each strain to confirm the temperature sensitivity of the mutant and are shown in the upper two panels of Fig. 2. Thus the MS regulatory mechanism, like the RNA control mechanism, responds to the absence of a full complement of aminocar-yl-tRNA synthetase reactions rather than the limited availability of a full complement of free amino acids.

Specificity of MS for Amino Acid Starvation—Neidhardt (26) has demonstrated that relaxed strains exhibit deranged RNA control only in response to amino acid starvation. When cell growth is limited by means other than amino acid starvation (such as carbon or nitrogen source limitation), the relaxed cells regulate their rate of RNA accumulation in a manner indistinuishable from stringent strains. Although $^R$ mutants have been shown in a previous section to be unable to produce MS
when amino acid starved, the question arises whether after carbon source limitation the slowing of RNA synthesis can occur without the appearance of MS. Accordingly a stringent-relaxed pair of strains (CP 78-CP 79) were assayed for the presence of MS as well as their protein and RNA content under conditions of different steady state growth rates as explained in Table III. The tabulated values for the RNA to protein ratio confirm the observation (26) that both strains had similarly lowered ratios when growth rates were limited through the use of succinate as a carbon source rather than glucose. Table III also indicates that neither the relaxed nor the stringent strain produced appreciable activities in MS compounds during steady state growth in succinate medium. Since the relaxed strain possesses a deranged amino acid control mechanism it is likely that in this strain other mechanisms regulate RNA synthesis during growth on succinate. The absence of appreciable labeling of MS I or MS II indicates that these compounds are probably unrelated to such processes of RNA regulation. Instead, this observation, taken with those of preceding sections, suggests that the appearance of label in the MS compounds is as specific for amino acid starvation as the RW' gene product is for the slowing of RNA synthesis. Experiments measuring the effect of transient changes in carbon sources are in progress.

**Kinetics of MS Regulation**—The experiments already described reveal that the appearance of MS can be correlated with three factors implicated in amino acid control of RNA biosynthesis. (a) The effects of the rel and rel' alleles of the RC gene, (b) the response to starvation for any amino acid, and (c) the effects of impaired aminoacyl-tRNA synthetase activity. Thus the MS compounds might represent a trivial effect of the cessation of RNA synthesis, as for example, the accumulation of nascent RNA chains. In order to obtain evidence bearing on this possibility, the kinetic data of MS appearance is compared with the cessation of RNA accumulation occasioned by amino acid starvation.

**Fig. 3** shows the results of an experiment which illustrates the over-all effects of methionine starvation and resupplementation on the appearance of MS. Cells of B333 were uniformly labeled with $^{32}$P, and incubated in a medium containing methionine at a concentration (2 µg per ml) which would be exhausted at a predictable cell density, O.D.750 = 0.25. Samples assayed for MS activity indicate that MS I and MS II appear when growth
Fig. 3. Strain B333 was grown in Tris-glucose minimal medium containing minimal amino acid requirements, filtered, washed, and resuspended as in Fig. 1 but in 2 µg per ml of methionine and at O.D.700 = 0.05 (32P at 5 x 10⁶ cpm per µmole of phosphate per 5 µl). Cell densities were monitored in a cold aliquot of culture and acid extracts were prepared at times indicated and chromatographed in 1.5 M KH₂PO₄ for determination of activity in MS I and MS II. Approximately 30 min after cessation of growth due to methionine exhaustion, methionine was added back to one aliquot (0.01 volume, 20 µg per ml, final concentration), but not to another, and acid extracts were prepared thereafter at intervals shown.

The activities in both MS compounds quickly rise to a level higher than the subsequently maintained plateau value. This overshoot has been observed in several experiments utilizing methionine exhaustion in B333 as well as with valine addition to strain CP 78; it suggests a complex regulation process. In order to determine whether amino acid addition to amino acid-starved cells leads to the disappearance of MS, methionine (20 µg per ml) was added back to an aliquot of the methionine-starved culture and MS assayed in the starved as well as the resupplemented aliquots. Fig. 3 indicates that the disappearance of MS I and MS II under these conditions is rapid, for both were virtually undetectable 1 min after methionine resupplementation.

These results indicate that a meaningful comparison of the timing of MS appearance with that of the effects of amino acid starvation on RNA synthesis requires a precisely timed onset of amino acid starvation with cells uniformly labeled with 32P. These conditions have been met with thiazole alanine addition to strain B333 as well as with valine addition to strain CP 78 (see Table II).

Fig. 4 shows the timing of the appearance of MS I and MS II after thiazole alanine addition to strain B333. The cells were grown, harvested, and resuspended as described in Table II with the exception that they were resuspended in media containing required amino acids. This suspension was divided into three aliquots. To one was added 32P (5.2 x 10⁶ cpm per µmole of phosphate per 5 µl); all three were grown for 30 min prior to the addition of thiazole alanine (50 µg per ml). The lower panel in Fig. 4 shows the results of assays for MS activity in the 32P-labeled culture performed on aliquots withdrawn and acidified at 10 sec intervals after thiazole alanine addition.

The remaining two aliquots were used to measure the time required for the onset of the stringent RNA response following thiazole alanine addition. For this purpose the amount of 3H-uracil incorporated into trichloroacetic acid-insoluble material during 20 sec pulses was employed instead of 32P incorporations for two reasons. The first reason is that measurements of trichloroacetic acid-precipitable 32P do not allow a precise identification of the inflection point in RNA accumulation curves (see Fig. 2). The second reason is that the conversion of uracil into trichloroacetic acid-precipitable material requires that uracil is phosphorylated (during the pulse) to the triphosphate level prior to its polymerization into RNA (9, 10, 27).

The upper panel of Fig. 4 gives the results of such pulse measurements after thiazole alanine addition as well as the control aliquot which received no thiazole alanine. Fig. 4 shows that MS I and MS II begin to appear 25 to 30 sec after thiazole alanine addition; the initial rates are 0.9 and 0.7 µmole of phosphate per O.D.700 per min, respectively. The slowing of labeled uracil incorporation into trichloroacetic acid-insoluble material during a 20 sec pulse occurs significantly later, about 110 sec after thiazole alanine addition. This observation of the appearance of MS prior to the onset of the stringent response indicates that MS appearance cannot be a consequence of this response. This conclusion is supported by similar measurements with strain CP 78 after isoleucine starvation was imposed by valine addition.

Fig. 4. A comparison of the time of onset of the stringent response and the appearance of MS after thiazole alanine (TA) addition to strain B333. The experimental procedure is described in the text. Lower, radioactivity appearing in MS I (open circles) and MS II (filled circles) is plotted as millimicromoles of phosphate per ml of culture. Upper, the trichloroacetic acid-insoluble radioactivity recovered after filtration of aliquots containing thiazole alanine (+TA) is plotted as open circles at mid-pulse times. The control was similarly subjected to tritiated uracil pulses, but without the addition of thiazole alanine (filled circles, -TA).
These observations do not rule out the possibility that the appearance of MS is a trivial consequence of an early reaction which later leads to the slowing of RNA synthesis. If this were the case the MS compounds might not have functional significance. Alternatively, if MS compounds do function to slow RNA accumulation, MS should disappear at least as rapidly as RNA accumulation resumes when amino acids are added back to an amino acid-starved culture.

Measurements which show that this prediction is satisfied are shown in Fig. 5. The lower panel is a semilogarithmic plot of the decay of MS I and MS II after methionine resupplementation of a B333 culture labeled with $^{32}$P$_1$ during a 30-min incubation in the absence of methionine. It can be seen that after methionine addition, MS II disappears very quickly and that the disappearance of MS I is only slightly less fast (after a lag of 10 sec). The initial rates of MS I and MS II disappearance are 5.4 and 6.6 mmoles of phosphate per O.D.100 per min, respectively.

The upper panel in Fig. 5 shows a measurement of the time required for the rate of RNA accumulation to resume after methionine resupplementation as measured by 20-sec uracil pulses similar to those described in Fig. 4. It is apparent that the onset of MS disappearance is at least as fast as the resumption of higher rates of uracil incorporation into RNA after the addition of methionine to methionine-starved cells. This observation still does not prove that MS is a regulator of RNA synthesis; MS might be broken down rapidly by processes quite unrelated to the amino acid control mechanism.

Effects of Chloramphenicol—The rationale for measuring the effects of chloramphenicol on MS activity in amino acid-starved

Fig. 5. Strain B333 was grown, harvested, and starved for methionine as in Fig. 1 for 30 min in the presence of $^{32}$P$_1$ (5.9 $\times$ 10$^6$ cpm per m mole of phosphate per 5 ml). At the times indicated aliquots were removed and acidified. Lower, the effect of methionine addition (20 $\mu$g per ml) on radioactivity appearing in MS I (open circles) and MS II (filled circles) was measured by chromatography. At the time of methionine addition the activity appearing in MS I (denoted MS I$'$) was 3.57 mmoles of phosphate per O.D.290, and in MS II (MS II$'$) was 1.08 mmoles of phosphate per O.D.290. Upper, trichloroacetic acid-insoluble radioactivity recovered after 20-sec pulses with $^3$H-uracil before and after the addition of 20 $\mu$g of methionine per ml.

Fig. 6. Strain B333 was grown and subjected to the same conditions as described in Fig. 5 to label MS I and MS II with $^{32}$P during a 30-min methionine starvation period (O.D.600 = 0.16). At the time indicated by the arrow, chloramphenicol was added at 10 $\mu$g per ml (open squares), 50 $\mu$g per ml (open circles), or 100 $\mu$g per ml (filled circles). Samples were assayed for MS I activity (middle) and MS II activity (lower). The values obtained are plotted as the ratio of the activity obtained at time (t) to the activity obtained just prior to chloramphenicol addition. The activities obtained prior to chloramphenic acid addition (MS I and MS II) are the same as in Fig. 5, as is the $^{32}$P$_1$ specific activity. For comparison the decay curves for MS I and MS II following methionine addition are shown (dashed line). Upper, a second aliquot of cells was subjected to the same conditions as the $^{32}$P-labeled portion, but not labeled with $^{32}$P$_1$. At time zero (after 40 min of starvation for methionine), tritiated uracil was added (2.5 $\mu$Ci per 0.71 pmole per ml of culture) to each of four aliquots and chloramphenicol added at 100 $\mu$g per ml (filled circles), 50 $\mu$g per ml (open circles), 10 $\mu$g per ml (open squares), or not added (filled squares). At 10-min intervals thereafter, 0.1 ml of each aliquot was precipitated with 5% trichloroacetic acid, and the acid-insoluble radioactivity was determined as a measure of RNA synthesis.
cells is 2-fold. First, a variety of evidence has implicated the effects of this inhibitor of protein synthesis in amino acid control of RNA synthesis (1, 3, 4). Low concentrations of chloramphenicol produce only partial inhibition of protein synthesis and no stimulation of RNA synthesis in amino acid-starved cells unless "catalytic" amounts of amino acids are also added (28). High concentrations of chloramphenicol which inhibit protein synthesis much more severely lead to stimulation of RNA synthesis in amino-acid-starved cells even without additional amino acid supplementations (29). These effects have been interpreted in terms of accumulations of amino acids resulting from protein turnover when protein synthesis de novo is partially or fully blocked by low or high concentrations respectively, of chloramphenicol (3). Thus the appearance of MS compounds can be correlated with the effects of chloramphenicol on RNA control.

The second reason for using chloramphenicol is to ascertain whether MS appearance is a consequence of the cessation of protein synthesis per se. Fig. 6 shows the effects of chloramphenicol addition at 10, 50, and 100 μg per ml to methionine-starved B333 which has been labeled with 32P, as in Fig. 5, as well as to a similarly prepared but unlabeled aliquot of cells to which tritiated uracil was added at the time of chloramphenicol addition. Assays for MS I and MS II were performed as in Fig. 5. For comparison, the disappearance of MS I and MS II after methionine addition is indicated by dashed lines. Fig. 6 indicates that the addition of chloramphenicol in concentrations high enough to increase the rate of RNA synthesis is also sufficient to cause the disappearance of MS I and MS II. At low concentrations (10 μg per ml) the rate of RNA synthesis is not increased, nor does it increase the rate of RNA synthesis is also sufficient to cause the disappearance of MS I and MS II. Therefore, the MS compounds are heavily labeled under conditions where the slowing of RNA synthesis occurs by means other than the amino acid RNA control mechanism (the slowing of RNA synthesis in a RCrel mutant growing on succinate as compared to glucose), the MS control mechanism does not produce MS compounds. These results indicate that the mechanism by which amino acids regulate RNA synthesis and MS are one and the same.

A trivial explanation of MS regulation is that it is a consequence of the slowing of RNA synthesis which is occasioned by amino acid starvation. This explanation is excluded since both MS compounds begin to appear before the rates of RNA accumulation begin to diminish when histidine starvation is imposed by thiamine sulfone addition.

Similarly, the appearance of MS compounds cannot be attributed merely to the slowing of protein synthesis, since relaxed mutants do not produce MS compounds after amino acid starvation, even though their rates of protein synthesis diminish in a manner indistinguishable from stringent strains. Furthermore, the addition of chloramphenicol at high concentrations (100 μg per ml) results in severe inhibition of protein synthesis (29) but causes the disappearance rather than the further accumulation of MS.

Earlier experiments have led to the suggestion that amino acid regulation of RNA synthesis occurs at the level of phosphorylation in the synthesis of RNA polymerase substrates (9, 10). More recent work shows that the controlling reaction is likely to involve the synthesis of purine ribonucleoside triphosphates (11). The effects of amino acid starvation on the formation of pyrimidine nucleoside triphosphates (10) and of non-nucleotidyphosphates (30) might be mediated by ATP availability (11). However, the MS compounds are heavily labeled under conditions where all other detectable acid-soluble phosphates show diminished 32P labeling (12). Thus the MS compounds might cause or reflect the alteration of some phosphorylating mechanism occasioned by amino acid starvation. It is also possible that the appearance of MS compounds is related to an energy shunting process.

It is clear from studies with strain EA 1 that MS II is a dispensable compound, for this strain shows the stringent RNA response to amino acid starvation even though it does not produce MS II. It is also evident that any hypothetical response to MS I is not an all-or-none phenomenon. EA 1 grown on minimal media supplemented with methionine gives rise to a 10- to 20-fold reduction in the rate of RNA synthesis when starved for methionine, and yet MS I is present at significant activities (0.4 μmole of phosphate per O.D.) and increases only about 5-fold upon methionine starvation (Table I).

The observations all together suggest that MS plays some role in regulating RNA synthesis. This role might be either active or passive. An active role would be that the MS compounds behave as functional inhibitors of RNA synthesis.

The distinction between active and passive roles is being approached experimentally with purified MS I preparations in two ways: (a) by determining whether MS addition is capable of inhibiting RNA synthesis in amino acid-starved plasmolysed...
cells carrying the relaxed allele of the RC gene; (b) by determining whether the phosphorylation of nucleotides to triphosphates is altered by MS addition. The chemical composition of MS I (purified from acid extracts of amino acid-starved stringent cells) is being determined; the results suggest that MS I is a periodate-insensitive, alkali-labile tetraphosphate of guanosine.

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