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Stable Ribonucleic Acid Synthesis in Stringent (*rel*$^+$) and Relaxed (*rel*$^-$) Polyamine Auxotrophs of *Escherichia coli* K-12

P. R. SRINIVASAN, DELANO V. YOUNG, AND WERNER MAAS

Department of Biochemistry, Columbia University, New York, New York 10032, and Department of Microbiology, School of Medicine, New York University, New York, New York 10016

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The relationship of polyamines to stable ribonucleic acid (RNA) synthesis under conditions of amino acid withdrawal or chloramphenicol treatment was examined with the use of a closely related *rel*$^+$, *rel*$^-$ pair conditionally incapable of synthesizing putrescine. Under conditions of polyamine starvation, the cellular sperimidine level fell to one-third to one-half of the value observed in putrescine-supplemented cultures and putrescine became undetectable; cadaverine was synthesized by both strains, but the relaxed strain, MA 252, accumulated less cadaverine per cell than its stringent twin, MA 254. Upon amino acid withdrawal, the stringent strain remained stringent whether starved of or supplemented with polyamines. Similarly, the relaxed strain was capable of making RNA either with or without polyamine starvation. On the addition of chloramphenicol or upon amino acid withdrawal in the relaxed strain, supplementation with spermidine had no effect on the initial rate of RNA synthesis, although RNA accumulation was greater in the presence of added spermidine. Spermidine added at the conclusion of RNA synthesis prompted additional synthesis, although preincubation with spermidine again had no effect on the initial rate. All forms of stable RNA species were made with polyamine supplementation. The present data appear to rule out the possibility that polyamines are primary causative agents in stimulating RNA synthesis, but rather suggest an indirect or secondary role for spermidine in which the polyamines "stimulate" stable RNA synthesis probably by relieving RNA product inhibition of RNA synthesis.

The ubiquitous occurrence of polyamines in living organisms has stimulated considerable interest in their biological function (2). As basic compounds, they are ideal candidates to serve as cations in structural units containing nucleic acids. However, other studies suggest that they may be involved in stimulating ribonucleic acid (RNA) synthesis in bacteria (3, 19) and deoxyribonucleic acid (DNA), ribonucleic acid, and protein synthesis in chicken embryos (18). Recently, Maas and his co-workers (13), as well as Morris and his associates (17), have isolated mutants of *Escherichia coli* K-12 which are conditionally incapable of synthesizing putrescine, and these mutants offer new approaches for elucidating the biological role of polyamines. In previous reports (6, 13, 23), it was observed that after starvation for polyamines in these mutants the generation time increased several-fold and the rates of protein and nucleic acid synthesis were greatly reduced. Addition of putrescine or spermidine to polyamine-starved cells set in motion a definite sequence of events; an early effect was the stimulation of protein synthesis followed by increases in stable RNA and DNA synthesis (23). These findings suggested that polyamines play an essential role in protein synthesis and (or) RNA synthesis.

To explore further a direct role of polyamines in RNA synthesis, we constructed a closely related pair of *rel*$^+$ and *rel*$^-$ strains which are also blocked in the synthesis of putrescine. It has been proposed that the intracellular level of polyamines, or more specifically the spermidine to putrescine ratio, controls the synthesis of stable RNA in relaxed (*rel*$^-$) cells during amino acid starvation and in stringent (*rel*$^+$) cells upon chloramphenicol addition (3, 19, 20). These early studies were conducted with an *E. coli* strain (15TAU$^-$) whose polyamine supply could

not be depleted. To achieve some of the effects observed by these investigators, they had to use rather large doses of exogenous polyamines. In this report, we have reexamined this proposal using the closely related pair of *rel*$^+$ and *rel*$^-$ strains which can be depleted of their polyamines. Our findings indicate that polyamines, either spermidine or putrescine, do not affect the initial rate of stable RNA synthesis but appear to increase the overall accumulation, suggesting that polyamines do not play a direct role in RNA synthesis as earlier proposed but rather alleviate certain conditions that inhibit RNA synthesis.

MATERIALS AND METHODS

Bacterial strains. Strain MA 254 requires threonine, leucine, thiamine, and either arginine and putrescine or ornithine for growth and is *rel*$^+$ (stringent control). Strain MA 252 has the same nutritional requirements and is *rel*$^-$ (relaxed control). Both strains are blocked in agmatine ureohydrolase (AUH$^-$) and have an arginine conditional requirement for putrescine. These strains were derived by transduction with phage 363 (a P1 derivative) prepared on strain NF 177 (8), a *rel*$^-$ mutant. The parent recipient strain was *arg* A$^-$; *met* B$^-$ and *arg* A$^+$ transductants were selected. This gene is co-transducible with the *rel* locus. MA 254 is a *rel*$^+$ transductant; MA 252 is a *rel*$^-$ transductant derived from this cross. Subsequently, the *arg* E mutation was introduced into both strains by selecting for *met* B$^+$.

Growth medium. The medium used contained salts medium (5) supplemented with 0.5% glucose, thiamine, biotin, leucine, threonine, methionine, serine, glycine, and either arginine or ornithine. All of the amino acids were present at a concentration of 100 μg/ml except for arginine (200 μg/ml). Thiamine and biotin were used at concentrations of 2 and 10 μg/ml, respectively. Methionine, serine, and glycine, although not required for growth, were included to enhance polyamine starvation. When arginine or ornithine was added, the growth medium was designated MMA or MMO, respectively.

Putrescine starvation procedure and measurement of RNA synthesis. The cells were starved of putrescine in the following manner (13, 23). Cultures grown overnight in MMO medium were refrigerated for 4 h; a sufficient inoculum was added to MMA medium to yield a 20-fold dilution and allowed to grow overnight at 37 C with aeration. These starved cells were then diluted to a density of 10^8 cells/ml with MMA medium and allowed to grow at 37 C until the density reached 2×10^8 cells/ml.

The cells were collected by centrifugation at 15 C, washed twice with salts medium (5), and resuspended in one-half of their original volume of MMA medium lacking leucine. Putrescine, spermidine, leucine, and chloramphenicol when added were present at a concentration of 100 μg/ml. The synthesis of RNA was followed with ^{14}C-uracil (0.5 μCi per 10 μg per ml) at 37 C. At the indicated intervals, samples of 0.2 ml were removed, precipitated with 10% trichloroacetic acid, and filtered through glass-fiber disks (Whatman GF/C). The filters were dried and counted in a toluene scintillation mixture in a Nuclear-Chicago scintillation counter.

Polyamine analysis. Polyamine-starved cells were harvested, washed once with salts medium (5), and resuspended in one-half their original volume of MMA medium to yield a final concentration of 3×10^8 to 4×10^8 cells/ml. Putrescine was added to a part of the culture, and the cells were shaken at 37 C with vigorous aeration. At intervals, samples of 5 to 10 ml were removed from both cultures, immediately centrifuged, and washed once with 0.9% sodium chloride at room temperature. The cells were resuspended in 0.5 to 1.0 ml of 0.2 M perchloric acid and kept at 4 C for 15 min. The perchloric acid extracts freed from the cells were analyzed for putrescine, spermidine, and cadaverine by the modified dansylation method described by Cohen et al. (4).

RESULTS

The strains MA 254 and MA 252, after overnight polyamine starvation as outlined earlier, grew poorly in MMA medium, with generation times of 274 and 456 min, respectively. In MMA medium supplemented with putrescine, the generation times were 82 and 187 min, respectively.

The intracellular polyamine content of both strains after polyamine depletion and after putrescine supplementation are shown in Table 1. To perform these measurements, both strains, after overnight starvation in MMA medium, were resuspended in fresh MMA medium and allowed to grow for 4 h. At this time, the cultures were divided into two; half of the cultures received putrescine. Samples were withdrawn from all cultures for polyamine analysis (see Materials and Methods), and the viable cell count was taken at each of the indicated times. In MMA medium, the level of putrescine was below detection for both strains and the intracellular spermidine present was approximately one-third to one-half of the level in the putrescine-supplemented cultures. Although cadaverine is synthesized by these cells in MMA medium, as previously reported (6), the relaxed culture produced significantly less cadaverine per cell than the stringent culture. After putrescine supplementation, cadaverine completely disappeared from both strains, whereas the spermidine level was found to increase gradually. The values for intracellular putrescine are considerably higher than those reported by other investigators (6, 17); this is probably due to putrescine contamination from the media.

Addition of putrescine or spermidine to polyamine-starved cells of the *rel*$^+$ strain, MA 254, caused a stimulation of RNA synthesis after a

TABLE 1. *Polyamine levels of strains MA 252 and MA 254 under various growth conditions*

Strain	Time of sampling (min)	MMA[a]		MMA + putrescine[b]	
		Spermidine	Cadaverine	Spermidine	Putrescine
MA 252 *rel*⁻	0	3.5[c]	1.3	—	—
	30	—	—	7.5	33.6
	60	4.6	1.3	—	—
	120	5.5	1.4	12.9	54.0
MA 254 *rel*⁺	0	4.8	2.4	—	—
	30	—	—	7.3	14.8
	120	3.2	3.4	10.9	29.0

[a] No measurable putrescine was present under these conditions.
[b] Cadaverine was absent under these conditions.
[c] Nanomoles per 10^9 cells.

lag period of 20 min, during which the rates of RNA synthesis in MMA medium and putrescine-supplemented MMA medium were identical (Fig. 1). In other experiments, the stimulation of RNA synthesis by putrescine after the initial lag was considerably more pronounced than in the results presented here. The increase in the RNA synthetic rate occurring after 60 min is, at least in part, due to an actual increase in the rate of cell division of putrescine-supplemented cells (23). As shown also in Fig. 1, after the inclusion of chloramphenicol at a level which effectively inhibits protein synthesis, RNA synthesis continued in all three media studied, MMA, MMA plus putrescine, and MMA plus spermidine. The initial rates of RNA synthesis appeared identical although the final level was higher in polyamine-supplemented media.

The effect of amino acid starvation on the rates of RNA synthesis in the *rel*⁺ strain is shown in Fig. 2. Withdrawal of leucine arrested RNA synthesis, and supplementation with either putrescine or spermidine failed to promote RNA synthesis under these conditions. As expected, addition of chloramphenicol gave the typical relaxed response in RNA synthesis, and the inclusion of putrescine or spermidine, although unable to alter the initial rates of RNA synthesis, again caused a greater accumulation of RNA at later times.

The *rel*⁻ strain, MA 252, was also examined for the effect of polyamines on RNA synthesis (Fig. 3). After the addition of putrescine to polyamine-starved cultures, there was a period of approximately 2 h during which the rates of RNA synthesis in starved and putrescine-supplemented cultures were identical. This is in

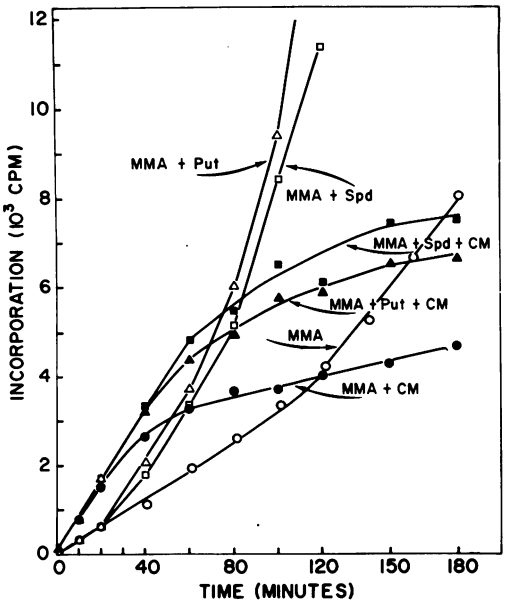

FIG. 1. *Effect of polyamine addition to polyamine-starved cells of strain MA 254 on the rates of RNA synthesis in the presence and absence of chloramphenicol. MMA + Put, MMA medium supplemented with putrescine; MMA + Spd, MMA medium supplemented with spermidine. CM, chloramphenicol (100 μg/ml). For details, see text.*

contrast to the situation with the *rel*⁺ strain, where the initial rates were identical for only 20 min. After this initial 2-h period, the rate of RNA synthesis was higher in the putrescine-supplemented medium. In the *rel*⁻ strain, starvation for the required amino acid, leucine, permitted the synthesis of RNA, and here again the initial rates in MMA medium and MMA medium supplemented with either putrescine or spermidine were identical, although the final level of RNA accumulation was higher in putrescine-supplemented cultures.

Since the initial rate of stable RNA synthesis in these experiments was not affected by polyamine supplementation, it appears that polyamines are needed only at a later stage of RNA synthesis. This interpretation raises a number of possibilities. Perhaps polyamines protect the newly synthesized RNA from degradation, thus raising its level of accumulation, or perhaps they are counteracting some inhibitor or inhibitors of RNA synthesis, thereby permitting RNA synthesis for a longer period of time. A third possibility, that polyamines simply require a prolonged period to enter the cell and perhaps interact with some cellular component, was also considered.

If rifampin was added to cells in the process of

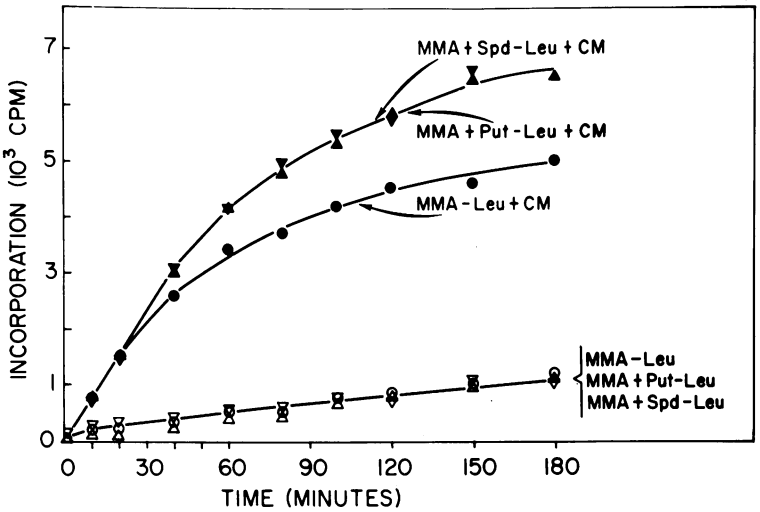

FIG. 2. *Effect of polyamine addition to polyamine-starved cells of strain MA 254 on the rates of RNA synthesis in the absence of leucine and in the presence and absence of chloramphenicol (CM).*

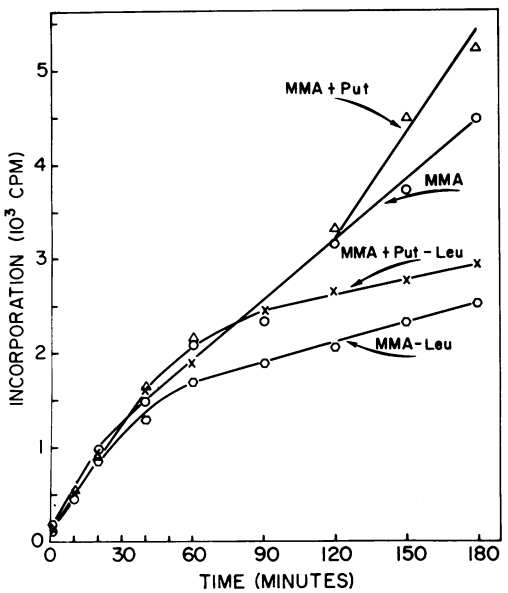

FIG. 3. *Effect of putrescine addition to polyamine-starved cells of strain MA 252 on the rates of RNA synthesis in the presence and absence of leucine.*

producing RNA, either to *rel*⁺ cells in the presence of chloramphenicol or to *rel*⁻ cells under amino acid starvation, RNA synthesis ceased immediately and degradation occurred. As shown in Fig. 4, the rate of degradation was independent of spermidine supplementation and, indeed, the final level of RNA was identical in both cases. A similar result with rifampin was obtained with *rel*⁻ cells during starvation for leucine (unpublished data). These results eliminate the possibility that polyamines protect the newly synthesized RNA from degradation.

In both the *rel*⁺ and *rel*⁻ strains, under conditions which lead to the accumulation of RNA, synthesis of RNA was nearly concluded by 80 min. If spermidine was added at this time to either strain, RNA synthesis again commenced after a short lag of 10 min (Fig. 4) and reached a level commensurate with the synthesis in cultures supplemented with spermidine at zero time. On the other hand, if spermidine was added 20 min prior to RNA synthesis, no immediate effect was seen; initially the rate was the same as that without spermidine followed by a higher level of RNA accumulation (unpublished data).

In considering the possibility that the incorporation of radioactive uracil in these mutants does not accurately reflect RNA synthesis, these experiments were repeated by directly measuring the total cellular RNA by the orcinol method (21). The same experimental results were obtained by this second method. In spermidine-supplemented cells, the synthesis of relaxed RNA with strain MA 252, or of chloramphenicol RNA with strain MA 254, was approximately 50 to 60% of the total cellular RNA found at the beginning of RNA synthesis. Although polyamine-starved cells accumulated much less RNA (60 to 80% of the level observed in polyamine-supplemented cells) and spermidine addition at 80 min caused renewed accumulation, cells never subjected to polyamine starvation by continuous cultivation in MMO medium synthesized relaxed or chloramphenicol

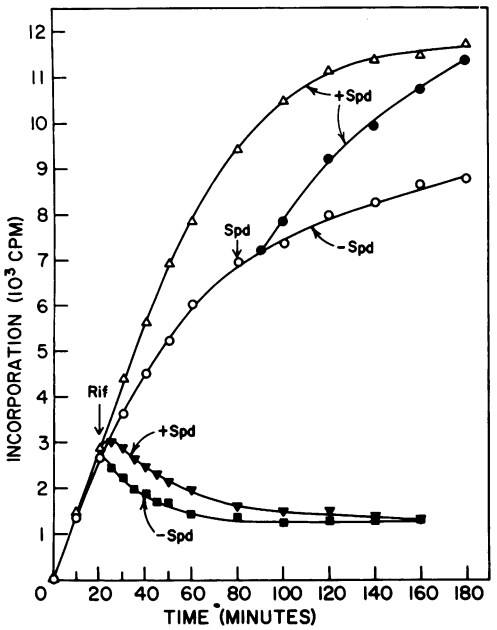

FIG. 4. *Effect of spermidine addition to polyamine-starved cells of strain MA 254 on the rates of RNA synthesis in the presence of chloramphenicol and in the presence of chloramphenicol and rifampin. After being washed with salts medium (5), the polyamine-starved cells were resuspended in MMA medium lacking leucine and containing chloramphenicol (100 μg/ml) and ^{14}C-uracil. The culture was divided into two; one portion received spermidine (100 μg/ml) at zero time. To determine the rates of RNA degradation, samples from the two cultures were introduced into separate flasks, kept at 37 C, containing rifampin (Rif; 50 μg/ml). To determine the effect of spermidine addition at a later period, spermidine (Spd, 100 μg/ml) was added to a portion of the polyamine-starved MMA-leu culture (i.e., in the absence of leucine and in the presence of chloramphenicol) at 80 min and the synthesis of RNA was again followed. Symbols: Δ, MMA-leu + CM + Spd; O, MMA-leu + CM; ●, MMA-leu + CM with Spd added at 80 min (arrow); ▼, MMA-leu + CM + Spd + rifampin (Rif) added at 20 min (arrow); ■, MMA-leu + CM + Rif (added at 20 min).*

col RNA to a final level intermediate between polyamine-starved and supplemented cells. The initial rate of RNA synthesis was not altered by the addition of spermidine to polyamine-starved cells of strain MA 254 in the presence of chloramphenicol or to polyamine-starved cells of strain MA 252 in the absence of leucine. However, the initial rate of RNA synthesis for cells in MMO medium was slightly faster (i.e., by 50%) than for polyamine-starved cells. The precise significance of this latter finding is not clear because of the vast physiological differences between these two cellular states.

Sucrose gradient centrifugation of the RNA products formed with or without polyamine supplementation indicated that if spermidine is added at zero time all forms of stable RNA species, ribosomal and transfer, are made in the same proportions as in the absence of polyamine supplementation (Fig. 5A). However, if spermidine is added at 80 min, slightly less 4*S* RNA is generated in the spermidine-supplemented medium.

DISCUSSION

Our experiments have attempted to determine the relationship of polyamines to stable RNA synthesis in the bacterial cell. Cohen et al. (3, 19, 20) have previously proposed that the intracellular level of polyamines, or, more specifically, the ratio of spermidine to putrescine in *E. coli*, governs the synthesis of stable RNA, especially as observed in "relaxed" cells during amino acid deprivation. They reported that the intracellular spermidine to putrescine ratio increases in "relaxed" cells during amino acid withdrawal (3, 19) and that the addition of high levels of exogenous spermidine (2,900 to 5,800 μg/ml) to stringent cells can cause these cells to accumulate RNA during starvation for an essential amino acid (20). Similarly, Mills and Dubin (16) have also shown that bactericidal concentrations of spermine (a polyamine not found in *E. coli*) can stimulate the initial rate of RNA synthesis in that organism. Unfortunately, the high levels of spermidine and spermine necessary in these experiments cast considerable doubt on the physiological significance of the results. The levels of spermidine used (29 to 58 times the levels used in our experiments) and of spermine (1,000 to 4,000 μg/ml) are known to inhibit effectively protein synthesis in exponentially growing cells (1, 7). This fact has been used by others (7) to explain the apparent stimulatory ability of spermidine as a sparing effect in which required amino acids are supplied by protein turnover. In the experiments reported here, these problems have been circumvented through the use of mutants whose polyamine levels could be depleted. In these strains, a comparatively low level of exogenous polyamine (100 μg/ml) is required to return depleted cells back to normal, rapid growth (Fig. 1; 23). Under these conditions, protein synthesis is not inhibited.

Our findings suggest that the polyamines are *not* the governing factors in the regulation of "relaxed" RNA synthesis as previously proposed. Spermidine, at exogenous levels capable of restoring rapid growth to polyamine-starved cells, does not affect the initial rate of RNA

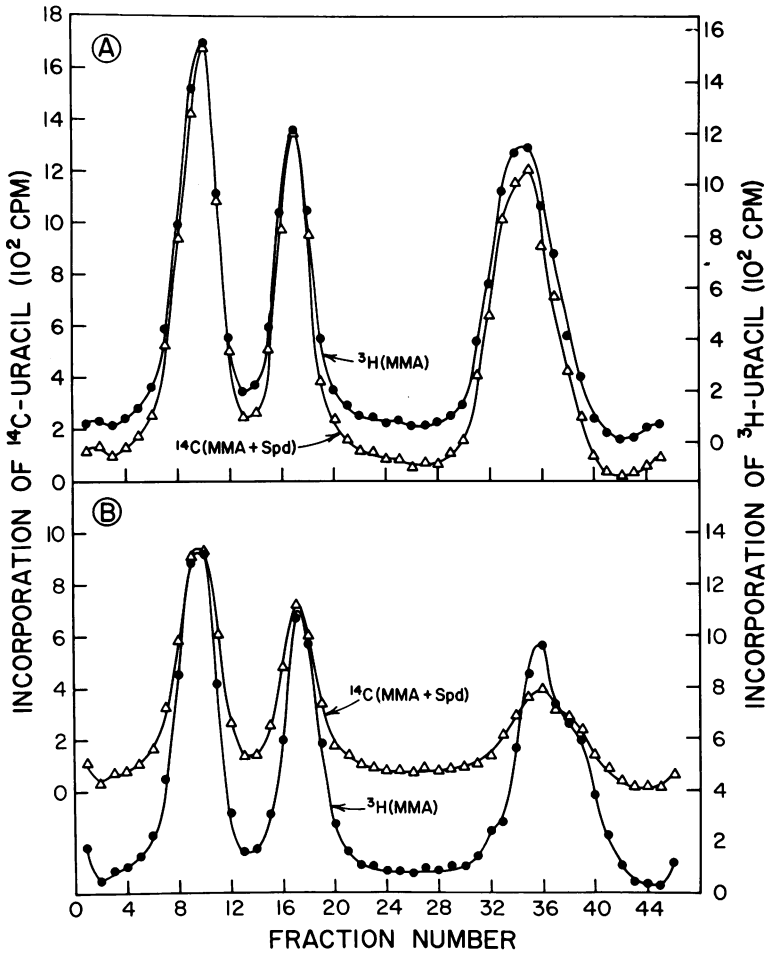

FIG. 5. *Sucrose gradient centrifugation profile of stable RNA species formed by polyamine-starved MA 252 cells upon withdrawal of leucine in the presence and absence of spermidine. Washed polyamine-starved cells were resuspended in MMA medium lacking leucine and divided into three portions: I, II, and III. One portion (I) received ^{3}H-uracil (1.5 μCi per 10 μg per ml) and the accumulated RNA was labeled for 180 min. Another portion (II) received ^{14}C-uracil (0.3 μCi per 10 μg per ml) and spermidine for 180 min. The third portion (III) received 10 μg of cold uracil/ml at 0 min. At 80 min, spermidine and undiluted ^{14}C-uracil (0.4 μCi/ml; 54 mCi/mmol) were added and the labeling was continued for another 100 min. Amounts of 5 ml each of I and II were mixed, and the RNA was extracted with phenol from lysed protoplasts (A in the figure); 5 ml of I and 5 ml of III were mixed, and the RNA profile from these cells is presented in B. Sucrose gradient centrifugation was performed on a 10 to 30% gradient in 0.01 M sodium acetate buffer, pH 5.0, with 0.1 M NaCl in an SW 25.1 rotor at 24,000 rpm for 41 h at 4 C.*

synthesis. During amino acid starvation, the *rel*$^{+}$ strain, MA 254, remains stringent with or without polyamine supplementation (Fig. 2), whereas, conversely, the *rel*$^{-}$ strain is capable of "relaxed" RNA synthesis during polyamine deprivation (Fig. 3). Furthermore, since these strains, when starved of polyamines, contain no detectable putrescine (Table 1), and since spermidine added alone to these starved cells already engaged in RNA synthesis can cause additional accumulation (Fig. 4), it appears that the *ratio* of spermidine to putrescine is not the governing factor responsible for the synthesis of stable RNA.

The possibility that the prolonged polyamine deprivation could also result in partial depletion of other cellular components required for RNA synthesis was considered. Our experimental results make this possibility extremely unlikely. As can be seen in Fig. 1, the addition of chloramphenicol to polyamine-starved cells leads to an immediate increase in the rate of RNA synthesis. This stimulation could not be possible if some component of the RNA syn-

thetic machinery were in limiting amounts and had first to be synthesized. This conclusion is further reinforced by the experiment depicted in Fig. 4, in which spermidine was added at 80 min to a chloramphenicol-treated culture of stringent cells starved for leucine. Under these circumstances, essentially no protein synthesis occurs, yet spermidine causes a sudden and large increase in the RNA synthetic rate. Also, when spermidine is added 20 min prior to chloramphenicol treatment of leucine-starved MA 254 cells, the initial rate of RNA synthesis is again unaffected, suggesting that the uptake of spermidine is not the limiting factor (unpublished data).

Although the polyamines do not appear to be the governing factor in the control of "relaxed" RNA synthesis, their presence does cause a greater accumulation of RNA in either the chloramphenicol-treated *rel*$^+$ strain or in the amino acid-starved *rel*$^-$ culture. This experimental fact raises the interesting question of how this is accomplished. From the rifampin experiment presented in Fig. 4, it appears that polyamines do not prevent RNA degradation. On the other hand, although a direct and active role for polyamines in RNA synthesis is not definitely ruled out by our experimental results, the fact that the initial rate of RNA synthesis is unaffected by putrescine or spermidine supplementation under all of the conditions studied (chloramphenicol treatment, amino acid starvation, or polyamine deprivation alone) makes this hypothesis very unlikely. The possibility that a decrease in the specific activity of the labeled precursor due to changes in the pool size caused by putrescine, as reported earlier (D. R. Morris, *In* Polyamines: their implications for effective cancer regulation, Nat. Cancer Inst. Symp., in press), might conceal any stimulation in the initial rate of RNA synthesis is excluded by the finding that spermidine added at the conclusion of RNA accumulation causes renewed synthesis after a comparatively short delay (Fig. 4). It is also known from recent work from our laboratories that chloramphenicol prevents any polyamine-promoted changes in the uracil pool (unpublished data).

Spermidine's inability to alter in a polyamine-starved strain the initial rate of synthesis and its ability to stimulate synthesis when it has concluded are results which are consistent with the interpretation that spermidine "stimulates" RNA synthesis indirectly by counteracting some inhibitor of this process. Since stimulation is not immediate but occurs only after some synthesis has occurred, it is likely that the inhibitor is a product of the reaction itself. RNA is known to inhibit RNA synthesis in vitro by binding to RNA polymerase (10, 11, 15, 22). Spermidine is also known to dissociate this complex (9, 10, 12, 14, 15) and thereby eliminate the product inhibition. A reasonable hypothesis envisions spermidine complexing with ribosomal RNA forming chloramphenicol or relaxed particles and thereby relieving the inhibition.

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