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Macromolecular Synthesis and Stability in Growth-Arrested BALB/c-3T3 Cells

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Concentrations of methylglyoxal bis-(guanylhydrazone) (mGBG) that inhibited serum-stimulated BALB/c-3T3 cells in late G_1 caused a marked inhibition of ^{3}H-leucine incorporation during a 20-min incubation. No decrease was observed in the incorporation of ^{3}H-uridine during a 20-min incubation; however, the amount of acid-insoluble ^{3}H-uridine in mGBG-treated cultures was decreased when the incubation period was longer than 20 min. The amount of the decrease in the accumulation of incorporated ^{3}H-uridine was directly proportional to the length of the incorporation time. Between 10 and 12 h after quiescent BALB/c-3T3 cells were serum-stimulated in mGBG no additional ^{3}H-uridine was accumulated. The stability of the incorporated ^{3}H-uridine, as determined by acid-insoluble radioactivity remaining after the addition of actinomycin D, was less in cells cultured in mGBG. Exogenous spermine or spermidine reversed the inhibition of ^{3}H-uridine accumulation in acid-insoluble material produced by mGBG as well as the decrease in stability of the incorporated ^{3}H-uridine in acid-insoluble material. The effects of mGBG on both the incorporation of ^{3}H-uridine and the stability of the incorporated ^{3}H-uridine can apparently be accounted for by an effect on ribosomal RNA.

Key words: methylglyoxal bis-(guanylhydrazone), cell cycle, RNA synthesis, RNA stability

Methylglyoxal bis-(guanylhydrazone) (mGBG) has been found to be a potent inhibitor of replication in several cultured cell lines. The growth of sparse 3T3 cells was decreased by addition of mGBG [1], as was the growth of primary rat embryo fibroblasts [2] and rat brain tumor cells [3]. The stimulation of various types of quiescent cells to reenter the cell cycle has also been shown to be inhibited by mGBG. The entry into S phase of bovine lymphocytes following stimulation by concanavalin A [4] as well as the initiation of DNA synthesis in serum-stimulated WI-38 cells [5] and BALB/c-3T3 cells [5,6] was decreased with mGBG treatment. mGBG is a well-characterized inhibitor of S-adenylmethionine decarboxylase activity [7] and has also been shown to lower the intracellular concentration of both spermine and spermidine in many different cell lines [2,5,8]. Whether mGBG can be biologically active by mechanisms not related to its effects on polyamines is not clear. It has been postulated that the growth inhibitory effects of mGBG are

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mediated solely by a decrease in polyamine concentrations [5], although this has not been shown to account for every system inhibited by mGBG [9].

We have previously described a late G_1 arrest point produced by mGBG in serum-stimulated density inhibited BALB/c-3T3 cells. It was observed that mGBG caused an inhibition in ^{3}H-leucine incorporation during a 20-min pulse at concentrations that caused no change in the rate of ^{3}H-uridine incorporation [6]. These data were similar to those reported by Holtta et al [8] who concluded that the inhibitory effects of mGBG might be due to a direct effect on protein synthesis. In this paper, we report that mGBG had effects on ^{3}H-uridine metabolism that were not reflected by the rate of incorporation of ^{3}H-uridine during short times of incubation. mGBG produced a decrease in the amount of total accumulation of ^{3}H-uridine incorporated into acid-insoluble material during extended incorporation periods. Although these effects appeared to be directly related to decreased cell-cycle transit, it is unclear if these alterations of RNA metabolism caused the inhibition of cell-cycle progression.

METHODS

Cell Culture

Stock cultures of BALB/c-3T3 cells were grown in 100-mm culture dishes in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum, 4 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2. To prepare cultures for experiments, approximately 5.0 $\times$ 10^4 cells in 2 ml medium were plated into 35-mm culture dishes. The medium was changed after three days and the cells were used after another four days.

Incorporation Studies

Either ^{3}H-leucine or ^{3}H-uridine was added to the culture medium at the appropriate times and after incubation as described for each experiment the medium was aspirated, the cells were washed with serum-free medium, and 2 ml of 5% TCA was added for two extractions (10-min each). After extensive washing with water, the precipitated material was solubilized in 0.5 ml 0.1 M NaOH and 1% SDS, and the amount of ^{3}H-labeled precursor was determined by liquid scintillation counting. Cell numbers per plate were determined for duplicate plates.

Materials

Antibiotics and horse serum were obtained from Grand Island Biologicals (Grand Island, NY), while calf serum was from Colorado Serum Co (Denver, Colo), and DME was from Flow Laboratories (Rockville, Md). Spermine and spermidine was purchased from Sigma Chemical Co (St. Louis, Mo) and methylglyoxal bis-(guanylhydrazone) was from Aldrich Chemical Co (Milwaukee, Wis). Radioactive isotopes were purchased from Becton-Dickinson (Orangeburg, NY). All other chemicals as well as the plastic tissue cultureware came from Fisher Scientific Co (Pittsburgh, Pa).

RESULTS

Earlier reports by Holtta et al [8] and ourselves [6] indicated that concentrations of mGBG that caused inhibition of cell-cycle traverse produced decreased rates of ^{3}H-leucine incorporation but not ^{3}H-uridine incorporation when measured

after short incubations (20-min). However, the amount of ^{3}H-uridine incorporated during a short interval primarily reflects nuclear RNA metabolsim, and it was possible that, although this parameter was not affected by mGBG, there were alterations in other aspects of RNA metabolism during incubation of the BALB/c-3T3 cells in medium containing mGBG. The data in Figure 1 are consistent with this hypothesis. Cells were incubated for either 15 or 120 min with ^{3}H-uridine at various times after the addition of either fresh calf serum or serum plus 30 μM mGBG. The amount of ^{3}H-labeled precursor incorporated into TCA-precipitable material was determined. There was no difference in the amount of uridine incorporated in cells treated with serum alone and those that were also treated with the inhibitor during the short incubations (15 min). These data were similar to those reported earlier [6,8]. However, at times after 3 h following serum stimulation, there were marked decreases in the amount of TCA-precipitable ^{3}H-uridine accumulated in the mGBG-treated cells cultures during the 120-min incorporation time.

To further investigate ^{3}H-uridine incorporation differences produced during long periods of incubation, experiments were performed where ^{3}H-uridine was added simultaneously to all cultures, in medium with and without mGBG, and then cultures were harvested after various intervals to determine the total incorporated ^{3}H-uridine accumulation. Figure 2 shows the accumulation of uridine over the first 6 h following stimulation with serum alone or with 30 μM mGBG. The accumulation of ^{3}H-uridine in serum-stimulated cultures was linear throughout the 6 h period. When cells were also treated with mGBG during serum stimulation, the ac-

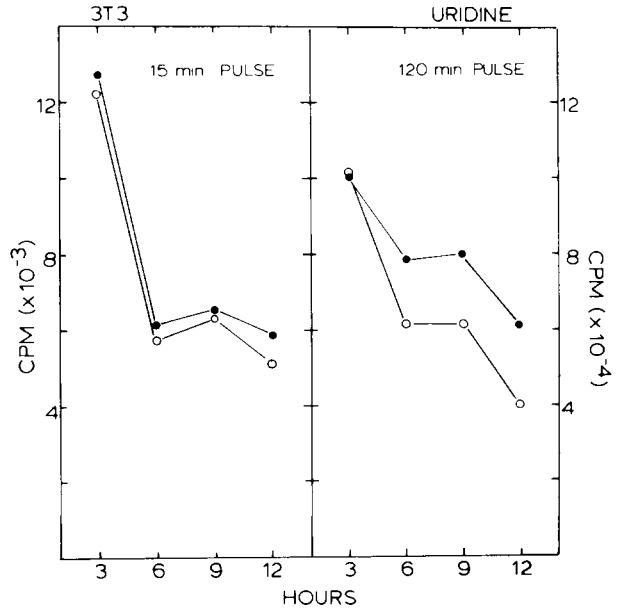

Fig. 1. Incorporation of ^{3}H-uridine as a function of time of incubation. At various times following stimulation of quiescent 3T3 cells with either serum alone (●) or serum plus 30 μM mGBG (○) duplicate cultures were pulsed for either 15 min (left panel) or 120 min (right panel) with 1 μCi/ml ^{3}H-uridine, and the amount of TCA-precipitable label was determined.

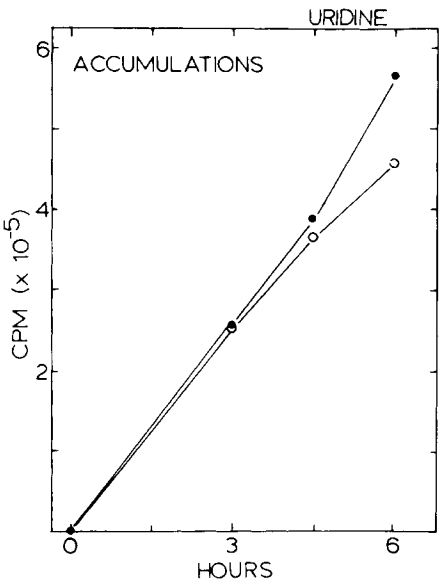

Fig. 2. Accumulation of ^{3}H-uridine over the first 6 h following serum stimulation. At 0 h cultures of quiescent 3T3 cells were stimulated either with 15% calf serum alone (●) or serum plus 30 μM mGBG (○). Also at 0 h, 5 μCi/ml ^{3}H-uridine was added to each culture dish, and at the indicated times, duplicate plates were harvested and the amount of incorporated label was determined.

cumulation of ^{3}H-uridine over the first 4–5 h was the same as in the cultures without mGBG; however between 4.5 and 6 h, there was a small decrease in ^{3}H-uridine accumulation in cultures containing mGBG. This was consistent with the data in Figure 1, since the ^{3}H-uridine incorporated in a 120-min incubation at 6 h after serum stimulation was decreased in cells treated with the inhibitor.

Figure 3 shows the accumulation of TCA-precipitable ^{3}H-uridine over the period from 6 to 12 h after the addition of serum alone or with 30 or 56 μM mGBG. As observed in Figure 2, the accumulation of ^{3}H-uridine was linear in the serum-stimulated cells over the duration of the experiment. However, when total ^{3}H-uridine accumulation was measured for times after 6 h following serum stimulation of the quiescent cells in the presence of mGBG, there were large decreases in total ^{3}H-uridine accumulation when compared with cultures that did not have mGBG present. At times between 6 and 10.5 h after serum stimulation, the accumulation of ^{3}H-uridine was linear in the mGBG-treated cells, although the accumulation was lower than that in the cultures stimulated with serum alone. Between 10.5 and 12 h, there was no net accumulation of ^{3}H-uridine in the cells treated with mGBG even though the amount incorporated in a 15-min incubation, during the same time period, was the same in cultures with or without mGBG (see Fig. 1).

In contrast to these data, when mGBG was added 6 h after the serum stimulation of quiescent BALB/c-3T3 cells, there was no decrease in the total accumulation of ^{3}H-uridine incorporated over the period from 6 to 12 h after serum addition (data not shown). These data are consistent with earlier observations that

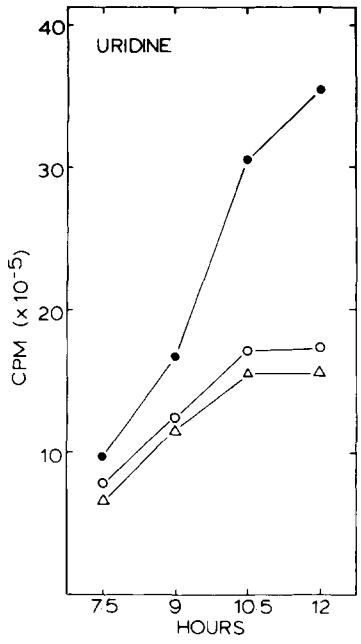

Fig. 3. Accumulation of ^{3}H-uridine over later times after serum stimulation. At 0 h either medium containing serum alone (●) or serum plus 30 μM (○) or 56 μM (△), mGBG was added to cultures of quiescent 3T3 cells. At 6 h, 5 μCi/ml ^{3}H-uridine was added to each culture dish, and at the indicated times, the amount of label incorporated into duplicate cultures was determined.

mGBG added 6 h after the stimulation of quiescent cells did not block the initiation of DNA synthesis [6]. These data indicate that the presence of the mGBG by itself was not sufficient to alter ^{3}H-uridine accumulation after serum stimulation. The data also show that the addition of mGBG at a time when only a small effect on cell-cycle traverse was observed also produced only minimal effects on uridine accumulation.

The addition of exogenous polyamines was shown to prevent the mGBG inhibition of cell-cycle traverse [6]. These experiments were performed in horse serum because it lacks a polyamine oxidase that is present in bovine serum [10]. The presence of exogenous spermine and spermidine in medium containing mGBG prevented the decreased ^{3}H-uridine accumulation during the period from 6 to 12 h after the addition of fresh horse serum (Table I). Thus, spermine (10 μM) and spermidine (10 μM) prevented the mGBG inhibition of G_0/G_1 traverse and the decreased ^{3}H-uridine accumulation after serum stimulation of density-inhibited BALB/c-3T3 cells.

Experiments were performed to determine the stability of the TCA-precipitable ^{3}H-uridine containing material in the presence or absence of mGBG. Cell cultures were incubated for 30 min with ^{3}H-uridine 6 h after the addition of fresh medium containing either serum or serum plus 30 μM mGBG. At the end of the 30-min incubation in ^{3}H-uridine, 5 μg/ml actinomycin D (ACT D) was added to each culture to inhibit new RNA synthesis and the amount of ^{3}H-uridine that could

TABLE I. Reversal of mGBG-Induced Decrease in ^{3}H-Uridine Accumulations

Treatment	cpm ^{3}H accumulated	
	− mGBG	+ mGBG
Control	42,631	17,742
Spermidine (10 μm)	39,320	43,915
Spermine (10 μm)	42,007	38,680

At 0 h quiescent density-arrested cultures were stimulated with fresh medium containing 15% horse serum, and various combinations of mGBG (30 μm) and either spermine or spermidine as indicated. At 6 h, 5 μCi/ml ^{3}H-uridine was added to each culture dish. After a 6 h incubation (at 12 h following stimulation), the plates were harvested and the total amount of TCA-precipitable counts accumulated was determined.

be precipitated by TCA after various times was determined. These results are shown in Figure 4. It can be seen that the amount of ^{3}H-uridine incorporated at the end of the 30-min incubation (at 0 h) was identical in cultures treated with or without mGBG. These results were similar to the results seen in Figure 1. After the addtion of ACT D the amount of TCA-precipitable ^{3}H-uridine decreased biphasically with a rapid initial drop followed by a slow steady decline (Fig. 4). There was no difference in the rate of the initial decline in ^{3}H-uridine precipitable counts between cultures with or without mGBG, although during the period from 2 to 6 h after the addition of ACT D (from 8 to 12 h after addition of serum), the rate of decline was somewhat greater in the cells cultured with mGBG than in those not treated with mGBG. Although the decreases observed after the longer incubations were small, the difference was extremely reproducible.

Since the later times in Figure 4 would predominantly represent stability of cytoplasmic RNA, a similar experiment was performed where more of the incorporated ^{3}H-uridine should be present in the cytoplasm from the beginning of the ACT D inhibition. To achieve this situation, the cells were incubated in ^{3}H-uridine during the first 6 h of exposure to either serum or serum plus mGBG, after which the amount of ^{3}H-uridine incorporated was approximately the same in each condition (Fig. 2). ACT D was then added, and the amount of ^{3}H-uridine present in TCA precipitable material was again determined after various lengths of time as shown in Figure 5. Using this experimental paradigm, a clear-cut difference between the stability of the incorporated ^{3}H-uridine was observed in the cultures with or without mGBG. The ^{3}H-uridine that had been incorporated into acid-insoluble material during the first 6 h of incubation disappeared much faster from cells growth in mGBG.

The data in Figure 6 provided indirect evidence that the differences in the stabilities of the incorporated ^{3}H-uridine were primarily effects on rRNA. In the experiments illustrated in Figure 6, the cells were stimulated at 0 h by transferring density-inhibited BALB/c-3T3 to fresh medium containing either serum alone or serum plus 30 μM mGBG. After incubating the stimulated cells for 5.5 h, 0.04 μg/ml ACT D was added to some cultures to inhibit preferentially rRNA synthesis [11]. The accumulation of ^{3}H-uridine was measured over the period from 6 to 12 h after the beginning of the serum stimulation. In cultures that did not receive ACT D, the patterns of accumulation were similar to those seen in Figure 3, with marked differences in ^{3}H-uridine incorporation between the cells treated with serum alone

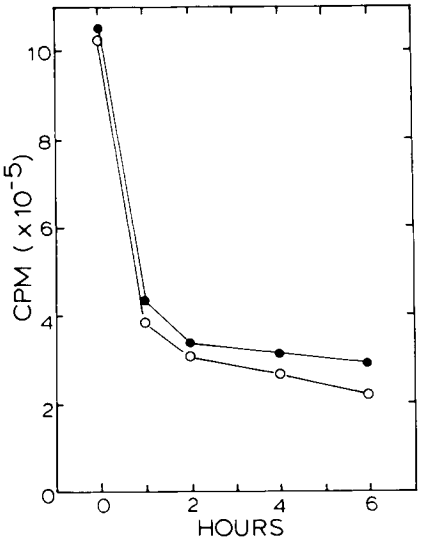

Fig. 4. Stability of preincorporated ^{3}H-uridine after a short pulse. After a 5.5-h exposure to fresh serum alone (●) or serum plus 30 μM mGBG (○), cultures were pulsed for 30 min with 2.5 μCi/ml ^{3}H-uridine. At the end of the pulse (0 h) 5 μg/ml ACT D was added to all the cultures and the amount of label present in a TCA precipitate was determined.

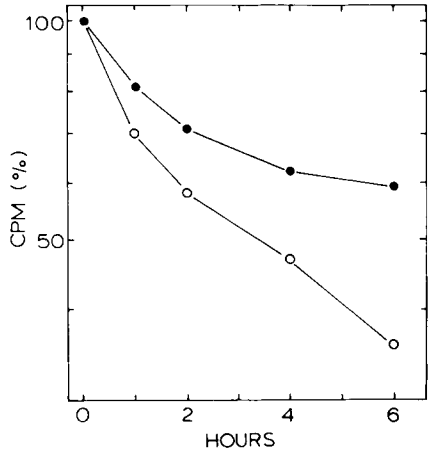

Fig. 5. Stability of preincorporated ^{3}H-uridine after a long pulse. After a 6 h exposure to 5 μCi/ml ^{3}H-uridine with either serum alone (●) or serum plus 30 μM mGBG (○), 5 μg/ml ACT D was added to cell cultures (at 0 h) and the amount of TCA precipitated label present was determined at various times.

and those that also received mGBG. However, when cells were treated with 0.04 μg/ml ACT D during the time of uridine incorporation, in order that only nonribosomal RNA was synthesized, the total amount of uridine accumulated was decreased by about 70%, and there was no difference in total ^{3}H-uridine accumulation in the cells that were treated with mGBG and those that were not.

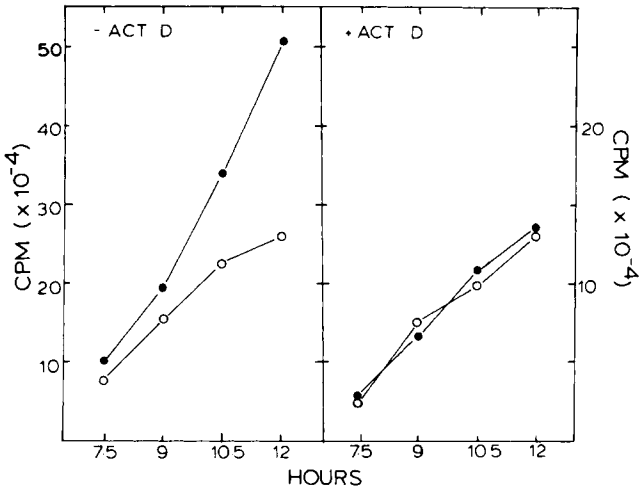

Fig. 6. Accumulation of ^{3}H-uridine in the presence of low levels of ACT D. At 0 h, quiescent cells were stimulated with either serum alone (●) or plus 30 μM mGBG (○). At 5.5 h, 0.04 μg/ml ACT D was added to some cultures (right panel), and at 6 h, all cultures received 5 μCi/ml ^{3}H-uridine. At the indicated times, duplicate cultures were harvested and the amount of label present in a TCA precipitate was determined.

Since the low levels of ACT D prevented the differences in total ^{3}H-uridine accumulation between cultures with and without mGBG, experiments were designed to again measure the differences in the stability of incorporated ^{3}H-uridine in cells cultured with or without mGBG. However, in these experiments, the incorporation of ^{3}H-uridine during the first 6 h after the serum stimulation of quiescent cells cultured with or without mGBG was performed in the presence of 0.04 μg/ml ACT D. After the 6 h of ^{3}H-uridine incorporation the ACT D concentration was raised from 0.04 μg/ml to 5.0 μg/ml. At the times indicated in Figure 7, the remaining TCA-precipitable ^{3}H-uridine was determined for both the cells cultured in either serum alone or in serum with 30 μM mGBG. The data in Figure 7 show when the cells had incorporated the ^{3}H-uridine in the presence of 0.04 μg/ml ACT D, there was no difference in the decrease in precipitable ^{3}H-uridine in cultures containing mGBG as compared with those cultures without mGBG.

DISCUSSION

The effects of inhibitors of cell-cycle traverse can provide important clues as to the biological processes that are critical to cellular proliferation. The data presented in this paper show that mGBG, a well-characterized inhibitor of proliferation in cultured fibroblasts [6,8], caused apparent alterations in the accumulation of ^{3}H-uridine that were not reflected by the rate of ^{3}H-uridine incorporated during a short incubation. The effect of mGBG on RNA metabolism was coincident with the inhibition in cell-cycle traverse.

There are several indications in the literature that RNA synthesis is critical for cell-cycle traverse. Inhibition of RNA synthesis by high dosages of ACT D inhibited proliferation as reviewed by Baserga [12]. The specific synthesis of ribosomal RNA

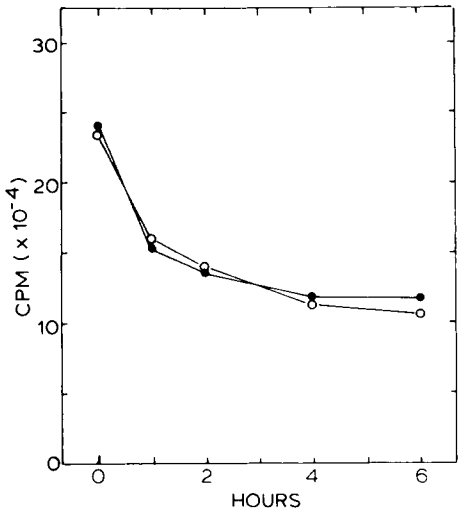

Fig. 7. Stability of ^{3}H-uridine incorporated during a long pulse in the presence of low levels of actinomycin D. After a 6 h exposure to 5 μCi/ml ^{3}H-uridine, 0.04 μg/ml ACT D and either 15% fresh calf serum alone (●) or plus 30 μM mGBG (○), 5 μg/ml ACT D was added (at 0 h), and the amount of label in a TCA precipitate was measured at the times indicated.

has been postulated to play a critical role in proliferation. Erfanova et al [13] showed that low concentrations of ACT D, which were used to specifically inhibit ribosomal RNA synthesis, caused an inhibition of cell cycle traverse. In addition, Darzynkiewicz et al [14] showed there was a direct correlation between the duration of G_1 in Chinese hamster ovary cells and the RNA content of the cell. These investigators use cytometry techniques that primarily measure ribosomal RNA. Their data confirm the postulated direct relationship between cell-cycle transit time and the accumulation of ribosomes proposed by Prescott [15]. A difference in ribosomal RNA metabolism in normal and SV-40 transformed human fibroblasts has also been described [16]. The data presented in this paper indicate that under conditions where mGBG inhibited cell-cycle transit, there was decreased stability of TCA-precipitable incorporated ^{3}H-uridine. Although experiments performed in the presence of low concentrations of ACT D indicated that the differences caused by mGBG on the stability of preincorporated ^{3}H-uridine was predominantly an effect on ribosomal RNA, direct measurements of RNA classes will be necessary to confirm this finding. It should be pointed out that although low concentrations of ACT D do preferentially inhibit ribosomal RNA synthesis [11], under some circumstances, other classes of RNA are also affected [17,18]. However, within the limitations of the techniques, our data agree well with the data previously reported that cell cycle traverse is directly related to ribosomal RNA content.

The relationship between a mGBG produced decrease in total ^{3}H-uridine accumulation and protein synthesis is not clear. A decreased rate of leucine incorporation was observed as early as 3 h after the addition of serum to quiescent cells in medium containing mGBG [6,8], while the effects on ^{3}H-uridine accumulation were not evident until 4.5 to 6 h following the stimulation of the cells. This might indicate that the effects on RNA metabolism were due to alterations in the levels of

certain proteins such as RNA methylating enzymes or ribosomal proteins. On the other hand, the decrease in protein synthesis might have resulted in decreased cellular ribosomes. A decreased synthesis of any of these proteins may have resulted in the decreased ^{3}H-uridine accumulation in mGBG-treated cells. The inhibition of total accumulation of ^{3}H-uridine seen in Figure 3 may, in part, reflect a balanced equilibrium between a decrease in unstable rRNA and lesser continuing incorporation into stable RNA as observed in Figure 3.

Whether mGBG was producing either the effects on cell-cycle traverse or RNA stability by effecting polyamine metabolism is not clear. mGBG has been shown to produce a marked decrease in both spermine and spermidine levels in 3T3 cells at concentrations that were used in these experiments [5]. In addition, the relationship between polyamines and RNA remains an object of wide speculation [9]. Exogenous spermine and spermidine reversed the effects of mGBG on both RNA metabolism and cell-cycle transit, but since the role of polyamines in the control of proliferation is not clear, further investigations will be necessary before this question can be resolved.

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REFERENCES

1. Rupniak HT, Paul D: Cancer Res 40:293, 1980.
2. Rupniak HT, Paul D: J Cell Physiol 94:161, 1978.
3. Heby O, Morton LJ, Wilson CB, Gray SW: Eur J Cancer 13:1009, 1977.
4. Fillingame RH, Sorstad CM, Morris DR: Proc Natl Acad Sci USA 72:4042, 1980.
5. Boynton AL, Whitfield JF, Isaacs RJ: J Cell Physiol 89:481, 1976.
6. Wharton W, Van Wyk JJ, Pledger WJ: J Cell Physiol 107:31, 1981.
7. Williams-Ashman HJ, Schenone A: Biochem Biophys Res Commun 46:288, 1972.
8. Holtta E, Pohjanpelto P, Janne Jr: FEBS Lett 97:9, 1979.
9. Tabor CW, Tabor H: Annu Rev Biochem 45:285, 1976.
10. Alarcon RA, Foley GE, Modest EJ: Arch Biochem Biophys 94:540, 1961.
11. Perry RP: Exp Cell Res 29:400, 1963.
12. Baserga R: "Multiplication and Division in Mammalian Cells." New York: Dekker, 1976.
13. Erfanova OI, Smolenskaya NI, Sevestyanova MV, Kurdyumova AG, ML: Exp Cell Res 58:401, 1969.
14. Darzynkiewicz Z, Evenson DP, Staiano-Coico L, Sharpless TK, Melamed: J Cell Physiol 100:425, 1979.
15. Prescott DM: "Reproduction of Eukaryotic Cells." New York: Academic Press, 1976.
16. Liebhaber SA, Wolf S, Schlessinger D: Cell 13:122, 1978.
17. Lindberg U, Peterson T: Eur J Biochem 31:246, 1972.
18. Penman S, Vesco C, Penman M: J Molec Biol 34:49, 1968.