rRNA Transcription Initiation Is Decreased by Inhibitors of the Yeast Cell Cycle Control Step “Start”*

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Inhibitors of the “start” regulatory step in the cell cycle of the yeast Saccharomyces cerevisiae are known by indirect studies to perturb RNA metabolism. We have investigated these effects further and show here by a pulse-labeling and quantitative hybridization procedure that pre-rRNA transcription was substantially decreased by five inhibitors of start but was transiently stimulated by the mating pheromone α-factor. Thus in contrast to the effects of the other start inhibitors, the inhibition of start by α-factor is unrelated to this aspect of biosynthetic activity. Mating factor treatment also stimulated the synthesis rate of poly(A)+ RNA. The start inhibitors o-phenanthroline and L-ethionine inhibited pre-rRNA transcription with little effect on poly(A)+ RNA synthesis rates. Northern analysis showed that all inhibitors of start also inhibited pre-rRNA transcript cleavage, a process that has been dissociated from the inhibition of start. Most inhibitors also affected ATP pool size. One inhibitor, o-phenanthroline, markedly induced the general control response.

For the budding yeast Saccharomyces cerevisiae the control of cell proliferation is exerted at the regulatory step termed “start” (Hartwell, 1974; Hartwell et al., 1974). Under most nutritional conditions the performance of start takes place when a cell has sufficient biosynthetic capacity to produce a viable daughter cell (Hartwell, 1974). Biosynthetic capacity in this situation is a function of cell mass, because the performance of start occurs only in cells that have acquired a threshold cell size or mass (Johnston et al., 1977). How cell mass is monitored for start is not clear.

Certain inhibitors have been shown to block cell proliferation by disabling the performance of start. For the mating pheromone α-factor this inhibition of start (Hartwell et al., 1974) is part of a response mediated by G-proteins (Dietzel and Kurjan, 1987; Miyajima et al., 1987; Jahng et al., 1988) that prepares the cell for conjugation (Hartwell, 1973). The inhibition of start by α-factor supersedes any biosynthetic control over start and takes place with little effect on global biosynthetic activity (Throm and Duntze, 1970). In contrast, other start inhibitors, within minutes after addition to cultures of growing cells, bring about significant biosynthetic inhibition. In most cases inhibition is significant for RNA synthesis with only modest effects on protein synthesis (Johnston and Singer, 1978; Singer et al., 1978; Singer and Johnston, 1979; Bedard et al., 1980). Indirect tests have indicated that in some of these start arrest situations the inhibition of rRNA or pre-rRNA production may account for most of the effects on RNA synthesis (Johnston and Singer, 1978; Singer et al., 1978; Singer and Johnston, 1979; Bedard et al., 1980).

In the study described here a quantitative hybridization analysis of pulse-labeled RNA (Veinot-Drebot et al., 1988) was used to measure, for yeast rRNA genes, the transcriptional perturbations caused by six different start inhibitors, including α-factor. With the exception of α-factor, each inhibitor caused rapid and substantial inhibition of pre-rRNA transcription. Northern analysis indicated that these inhibitors also slowed cleavage of the pre-rRNA primary transcript (Udem and Warner, 1972), a perturbation known to be without influence on the control of yeast cell proliferation (Johnston and Singer, 1980). Only one inhibitor markedly induced the general control response (Hinnebusch, 1988). A uniform correlation was therefore found between inhibition of pre-rRNA transcription and inhibition of start.

**MATERIALS AND METHODS**

**Strains and Media**—Cells of S. cerevisiae strain GR2 (MATα his6 ura1; Johnston and Singer, 1978) were grown with gyration shaking at 22 °C in YNB minimal medium (Johnston et al., 1977) supplemented either with histidine and uracil (40 μg/ml) for transcription rate studies or with histidine, uracil, adenine, arginine, leucine, lysine, and tryptophan for Northern analysis. Cell growth was monitored with an electronic particle counter (Coulter Electronics, Hialeah, FL; Hartwell, 1970).

**Plasmids and Plasmid Preparation**—Plasmid pLD11 (Veinot-Drebot et al., 1988), the ETS probe, contains the 665-base pair XmaII-HindIII yeast rDNA fragment from plasmid pJHC11 (Cramer et al., 1977; a gift from J. Cramer, Agrigenetics Corp., Madison, WI) substituted into pUCS (Vieira and Messing, 1982). The ITS probe was M13mp18-rD4+ (Veinot-Drebot et al., 1988; a gift from J. R. Warner, Albert Einstein College of Medicine, Bronx, NY). The HIS4 and URA3 probe was plasmid pH1 (a gift from A. Hinnebusch, NIH). Plasmids were prepared as described (Messing, 1983).

**RNA Labeling, Extraction, and Quantitation**—Procedures for RNA labeling were as described (Veinot-Drebot et al., 1988). Cells were grown for several generations in medium containing [35S]uracil (0.01 μCi/ml; Du Pont-New England Nuclear) to label stable RNA species and thus facilitate quantitation of RNA yields. For pulse labeling, a 2-ml sample of the [35S]-labeled culture, at 3.5 × 10⁵ cells/ml, was incubated with [3H]adenine (100 μCi/ml; 21.6 Ci/mmol; Du Pont-New England Nuclear) for a 2-min period and then diluted with 3 volumes of ice-cold water and subjected immediately to RNA extraction by the following modification of published procedures (Udem and Warner, 1972; Li et al., 1985). Cells were rapidly harvested by centrifugation, suspended in 0.5 ml of NETS buffer (Udem and Warner, 1972), and vortexed along with 1 ml of acid-washed baked
Hybridization of \[^{3}H\]RNA to immobilized DNA was carried out in capped plastic scintillation vials at 40 °C for 69 h. Each vial contained 0.5 ml of hybridization buffer (Kim and Warner, 1983), one filter piece containing pLD11 DNA, one filter piece containing pUC5 DNA, and 5 μg of total \[^{3}H\]RNA. Following hybridization, filters were washed at 40 °C first with 2 × SSC containing 40% deionized formamide, and then 10 times with 2 × SSC containing 0.2% sodium dodecyl sulfate. Filters were then washed at 22 °C once with 2 × SSC and finally twice with 85% ethanol. Bound radioactivity was measured by liquid scintillation in a toluene-based mixture. The extent of hybridization determined in this way increased linearly up to at least 75 μg of total RNA per reaction. The routine use of 5 μg of total RNA per reaction ensured DNA excess conditions.

**ATP Pool Specific Activity Determination**—Cells were grown for several generations in medium containing \[^{32}P\]PO₄ (2 μCi/ml; Du Pont-New England Nuclear) to uniformly label cellular ATP. From cultures in the range of 2.5 × 10⁶ cells/ml, 1-ml samples were incubated for 2 min at 30 °C. Prelabeled cells were then quickly harvested by centrifugation and immediately extracted at 4 °C overnight with 50 μl of 1 M formic acid. The contents of clarified extracts were resolved, along with nucleoside triphosphate standards, by thin layer chromatography (Cashel et al., 1969) on impregnated 0.1-mm cellulose MN300 polyethyleneimine plates (Brinkmann Instruments). The \[^{32}P\]/P ratio in ATP was determined by liquid scintillation counting (in a mixture of 1.5 ml of water and 10 ml of universal liquid scintillation mixture) of material from the center of the ATP spot. GTP was not routinely quantitated; preliminary work showed that insignificant \[^{3}H\]GTP was formed under these labeling conditions.

**Hybridizable ETS RNA versus pulse-labeling time.** Proliferating cells prelabeled with \[^{3}H\]oracil were pulse-labeled with \[^{3}H\]adenine for various times. The resultant total RNA was extracted and hybridized to immobilized plasmid pLD11 DNA coding for ETS pre-rRNA sequences.
short half-life of ETS RNA but allows quantitation of labeled transcripts with minimal interference from ETS degradation.

No matter what the rate of RNA synthesis, conditions that alter the size of the ribonucleoside triphosphate pools will as a consequence affect the rate of incorporation of exogenous labeled precursor molecules into RNA and thus affect the apparent rate of transcription. Therefore pool size was taken into consideration; each time RNA was pulse-labeled with [3H]adenine for hybridization, the relative specific activity of the total cellular ATP pool was also determined for a parallel culture that had been labeled continuously with [35P] (see "Materials and Methods"). The changes in relative specific activity (defined as the ratio of [3H]ATP from pulse labeling to [35P]ATP from continuous long term labeling) were used to correct for pool effects on label incorporation. (Parallel cultures were necessary because the procedure used to pulse label ATP was incompatible with that used to label RNA to high specific activity for hybridization; see "Materials and Methods."). At each labeling time the relative rate of label incorporation into total RNA was also determined for each parallel culture to verify similar responses of the parallel cultures to the same treatment.

Transcriptional Effects of α-Factor Treatment—Cells treated with the mating pheromone α-factor continue to enlarge as mass accumulation continues in the absence of bud formation (Throm and Duntle, 1970). Accordingly, it was found by pulse labeling that total RNA synthesis rates actually increased in α-factor-treated cells (data not shown). Part of this increase was due to increased rates of synthesis of mRNA, represented in the poly(A)+ RNA fraction (Fig. 2A). Mating factor treatment induces the expression of certain protein-coding genes (Hagen and Sprague, 1984; Hartig et al., 1986; Jahng et al., 1988; Kronstad et al., 1987; McCaffrey et al., 1987; Nakayama et al., 1985; Orlean, 1987; Trueheart et al., 1987), which could account for at least part of this increased rate of poly(A)+ RNA synthesis. In these α-factor-treated cells there also was significantly increased transcription initiation for the rRNA genes, as indicated by increased hybridization of pulse-labeled RNA to immobilized ETS DNA (Fig. 2A). The rRNA genes are therefore included in the set of genes that are modulated by mating factor treatment.

Transcriptional Effects of Other Start Inhibitors—In contrast to the continued transcription seen upon treatment with α-factor, there was significant inhibition of transcription caused by every other inhibitor of start. More specifically, each inhibitor produced substantial inhibition of pre-rRNA transcription.

The effects on pre-rRNA transcription caused by these inhibitors are exemplified by the effects of the start inhibitor L-ethionine (Singer et al., 1978). Most of the transcriptional effects following L-ethionine treatment were limited to inhibition of transcription of the rRNA genes (Fig. 2B). There were only minor effects on the transcription of poly(A)+ RNA (Fig. 2B), consistent with the continued protein synthetic activity of cells treated with L-ethionine (Singer et al., 1978). Therefore L-ethionine is an inhibitor of rRNA gene transcription.

As shown in Fig. 2C, even more substantial inhibition of pre-rRNA transcription was found for the start inhibitor o-phenanthroline (Johnston and Singer, 1978). Total RNA synthesis was also inhibited, but little of the o-phenanthroline-mediated decrease in the total RNA synthesis rate could be ascribed to inhibition of mRNA synthesis; in fact, the rate of synthesis of RNA in the poly(A)+ fraction was actually increased 1.7-fold at 15 min after inhibitor addition (data not shown), and protein synthesis has been shown to continue in o-phenanthroline-treated cells (Johnston and Singer, 1978). Furthermore, the inhibition of total RNA synthesis, of which about half is pre-rRNA (Shulman et al., 1977), was only half that of pre-rRNA transcription (data not shown), indicating that virtually all of the decrease in total RNA synthesis rate was accounted for by the inhibition of pre-rRNA transcription.

The inhibitory effects produced by the start inhibitor 8-hydroxyquinoline (Johnston and Singer, 1978) were less specific. Although there was rapid and remarkable inhibition of the rate of transcription of the rRNA genes (Fig. 2D), there was also similar inhibition of the synthesis rate for total RNA (data not shown), implying that mRNA synthesis was similarly inhibited.

Two other inhibitors of start also produced more general inhibition of transcription, similar to the effects of 8-hydroxyquinoline. Nalidixic acid (Singer and Johnston, 1979) inhibited RNA gene transcription efficiently (Fig. 2E) but also inhibited the synthesis of poly(A)+ RNA to a significant extent (Fig. 2E). These effects are consistent with the degree of inhibition of 35 S pre-rRNA accumulation and of amino acid incorporation for nalidixic acid-treated cells (Singer and Johnston, 1979). Sinefungin (Li et al., 1984) had general inhibitory effects on transcription that were less severe than those of nalidixic acid. Not only was RNA gene transcription inhibited by sinefungin (Fig. 2F), but poly(A)+ RNA synthesis was inhibited (Fig. 2F) to a degree consistent with the reported inhibition of amino acid incorporation (Li et al., 1984).

Inhibition of Pre-rRNA Cleavage—The assay described above indicates the rate of transcription of the rRNA genes but not the fate of the pre-rRNA transcript after synthesis. Therefore the ETS sequences used to measure transcription rates were also used in Northern analysis to measure levels of pre-rRNA transcript in inhibitor-treated cells. From the levels of 35 S pre-rRNA and the extent of inhibition of pre-rRNA transcription quantified above, the effectiveness of the initial cleavage step in the conversion of 35 S pre-rRNA to mature rRNA molecules was deduced.

In cells treated with α-factor the amounts of 35 S pre-rRNA present in cells increased somewhat (Fig. 3A). This increase is consistent with the increased rate of pre-rRNA transcription in α-factor-treated cells (Fig. 2A). Analogous increases.

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**Fig. 2. Effects of start inhibitors on rates of transcription and ATP pool specific activities.** Proliferating cells were treated at time zero with start inhibitors, and at the indicated times cells were pulse-labeled for 2 min periods with [3H]adenine. Rates of label incorporation into ETS sequences of pre-rRNA (panels A–F, closed circles) and into poly(A)+ RNA (open circles) were determined after correction for changes in ATP pool specific activity (panels G–L). All values are expressed relative to initial values, and each point is the average of duplicate determinations. The start inhibitors were as follows: panel A and G, α-factor; panels B and H, L-ethionine; panels C and I, o-phenanthroline; panels D and J, 8-hydroxyquinoline; panels E and K, nalidixic acid; panels F and L, sinefungin.
inhibitors were: α-F, α-factor; HQ, 8-hydroxyquinoline; Eth, L-ethionine; NaI, naladixic acid; OP, o-phenanthroline; SF, sinefungin.

in the levels of 35S pre-rRNA were also seen in cells treated with the start inhibitors o-phenanthroline and 8-hydroxyquinoline (Fig. 3A) but for a different reason. In these situations the increased levels of 35S pre-rRNA, seen in parallel with significant inhibition of pre-rRNA transcription (Fig. 2, C and D), do not reflect increased rates of synthesis but instead must result from significantly inhibited rates of initial cleavage of pre-rRNA by these start inhibitors. These findings show that the effects on pre-rRNA metabolism of both o-phenanthroline and 8-hydroxyquinoline are more extensive than just the inhibition of pre-rRNA transcription.

The inhibition of initial cleavage of pre-rRNA brought about by these two inhibitors must approximate the degree of inhibition of pre-rRNA transcription initiation. This conclusion stems from kinetic considerations. The intact 35S pre-rRNA molecule is produced in great quantity but has a very short half-life (approximately 0.5 min under these conditions; Veinot-Drebot et al., 1988), so that for these molecules even a slight perturbation in the relationship between the rate of synthesis and the rate of loss (initial cleavage) would be readily evident as a large change in the amount of intact pre-rRNA. The modest increases in the levels of 35S pre-rRNA brought about by treatment with o-phenanthroline or 8-hydroxyquinoline and the relative constancy of this increase over time (Fig. 3A) suggest that under these start arrest conditions the degree of inhibition of initial cleavage approximates the degree of inhibition of transcription initiation seen in Fig. 2.

In contrast to the comparable effects on pre-rRNA transcription and initial cleavage caused by o-phenanthroline and 8-hydroxyquinoline, the effects on pre-rRNA cleavage brought about by the other start inhibitors were more severe. For example, Northern analysis indicated that L-ethionine treatment markedly impeded the cleavage of pre-rRNA at many steps. Fig. 3A shows that 35S pre-rRNA molecules accumulated significantly in cells treated with L-ethionine, indicating that the initial cleavage step that removes ETS sequences from the pre-rRNA transcript was inhibited to an even greater degree than the already substantial inhibition of transcription of the RNA (Fig. 2B). Furthermore, analysis of the same RNA blot with another probe specific for sequences within the first ITS in the pre-rRNA transcript indicated that ITS-containing intermediates of pre-rRNA cleavage also accumulated in L-ethionine-treated cells (Fig. 3B). A similar pattern of accumulation of both 35S pre-rRNA and ITS-containing intermediates was seen for cells treated with the start inhibitor sinefungin (Fig. 3, A and B). An accumulation of 35S pre-rRNA was also noted for cells treated with the start inhibitor naladixic acid (Fig. 3A).

Therefore each inhibitor of the start regulatory step for cell proliferation that also inhibits the transcription of pre-rRNA also inhibits at least the initial cleavage step in the conversion of this transcript into mature rRNA. This inhibition of pre-rRNA cleavage, by stabilizing ETS RNA, also leads to overestimation in the assay described here of the rate of ETS RNA synthesis.

**Induction of the General Control Response**—In addition to effects on pre-rRNA transcription, other transcriptional effects have been associated with some of these start inhibitors. L-Ethionine has been shown (Wolfner et al., 1975; Penn et al., 1983) to be an amino acid analog that can induce the general control response, a regulatory system governing the expression of genes involved in the biosynthesis of amino acids (Hinnebusch, 1988). Certain conditional mutations that affect general control also inhibit start (Wolfner et al., 1975) or bring about unbudded cell arrest (Harashima et al., 1987), suggesting the possibility that the general control response may be involved in the start inhibition produced by some of these inhibitors. For this reason the general control response was monitored in cells treated with inhibitors at concentrations that caused start arrest.

One characteristic feature of the general control response is induction of the HIS4 gene, as indicated by increased levels of HIS4 mRNA (Donahue et al., 1983; Penn et al., 1984). Therefore HIS4 mRNA levels were determined after the addition of inhibitors and compared to levels of the URA3 transcript; the URA3 gene is not subject to general control
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(Silverman et al., 1982; Penn et al., 1983). Transfer of the histidine auxotrophic strain used here from supplemented to histidine-free medium caused the expected increase in HIS4 transcript levels (data not shown). For cells of the same strain in the same supplemented medium, the inhibitors at concentrations that cause start arrest produced differing degrees of activation of the general control response. The inhibitor o-phenanthroline caused significant induction of HIS4 transcript levels with little effect on URA3 transcript levels (Fig. 4), while 8-hydroxyquinoline had no effect on HIS4 transcript levels (Fig. 4). Other inhibitors, including L-ethionine and nalidixic acid, caused more modest and delayed increases in the relative levels of HIS4 mRNA (Fig. 4), effects similar to but more pronounced than those produced by α-factor treatment (Fig. 4).

These observations sustain the possibility that the general control response could be involved in the start arrest produced by o-phenanthroline treatment. In contrast, for L-ethionine and the other inhibitors at concentrations that bring about start arrest this involvement may not be significant.

**DISCUSSION**

The mechanisms that make the performance of start and thus all the activities of yeast cell proliferation responsive to the biosynthetic status of the cell are subject to investigation through the use of inhibitors of start. The “natural” inhibitors of start, the mating pheromones, apparently uncouple start from the biosynthetic status of the cell, leaving these activities generally unaffected (Throm and Dunetz, 1970; Fig. 2A). Thus other start inhibitors must be used to delineate biosynthetic activities that influence the performance of start. Some of these inhibitors have been studied here.

Many start inhibitors, identified solely by effects on cell proliferation, also affect RNA metabolism (see the Introduction). Here we document that, in fact, each start inhibitor affects expression of the rRNA genes. The mating pheromone α-factor transiently increased transcription of pre-rRNA. In contrast, every other inhibitor of start inhibited the transcription and the cleavage of pre-rRNA. It is notable in this context that heat shock, a treatment that causes transient start arrest (Johnston and Singer, 1980), also causes transient inhibition of both transcription (Veinot-Drebot et al., 1989) and cleavage (Shulman and Warner, 1978) of pre-rRNA in yeast. For each inhibitor the kinetics of inhibition of pre-rRNA transcription and cleavage paralleled the RNA inhibition kinetics determined earlier by indirect means. It was also shown that in addition to the effects on pre-rRNA, most of these inhibitors also had other effects on RNA metabolism; o-phenanthroline induced the general control response while 8-hydroxyquinoline, nalidixic acid, and to some extent sifinugen inhibited poly(A)^+ RNA synthesis generally. Furthermore, a previous study showed that sifinugen retards the methylation of rRNA, leading to a differential loss of undermethylated 18 S rRNA (Li et al., 1985). Each of these inhibitory effects reflected in perturbed RNA metabolism precedes or coincides with the inhibition of start and thus could in principle participate in start regulation.

Despite the correlations just described, some of these inhibitory effects can be eliminated from consideration as possible influences on the performance of start. For example, other studies show that inhibition of the cleavage of pre-rRNA is not itself responsible for start arrest. This conclusion is evident from the effects of an rna2 mutation in yeast (Johnston and Singer, 1980). Temperature-sensitive mutations in the RNA2 gene block intron removal from primary transcripts (Roshbash et al., 1981; Bromley et al., 1982; Teem et al., 1983) that code for ribosomal proteins (Roshbash et al., 1981; Fried et al., 1981). The resultant rna2-mediated inhibition of the synthesis of ribosomal proteins also inhibits the synthesis of proteins necessary for proper cleavage of the pre-rRNA primary transcript. As a consequence there is significant inhibition of pre-rRNA cleavage (Warner and Udem, 1972), but this inhibition does not affect the performance of start; rna2 mutant cells initiate new cell cycles at the nonpermissive temperature (Johnston and Singer, 1980). This ability of cells blocked in pre-rRNA cleavage to perform start suggests that the inhibition of pre-rRNA cleavage caused by the start inhibitors studied here (Fig. 3) does not affect start.

The induction of the general control response by o-phenanthroline is also probably not involved in either the inhibition of pre-rRNA transcription or the start arrest caused by this inhibitor. In other situations that induce the general control response such as growth of prototrophic cells in certain supplemented minimal media (summarized by Hinnebusch, 1988), treatment of cells with amino acid analogs (Wolfner et al., 1975; Hinnebusch and Fink, 1983; Penn et al., 1983; Lucchini et al., 1984), or the presence of gcd mutations under permissive conditions (Wolfner et al., 1975; Harashima and Hinnebusch, 1986), there is ongoing cell proliferation, indicating that rRNA production and the performance of start continue. Conversely, inhibition of pre-rRNA transcription and cleavage does not necessarily induce the general control response, as shown by the effects of 8-hydroxyquinoline (Fig. 4). In this particular situation the absence of HIS4 induction was not simply a result of the general inhibition of poly(A)^+ mRNA synthesis caused by 8-hydroxyquinoline treatment (see above). These inhibited cells retain significant biological potential, as indicated by the efficient conjugation of haploid yeast cells treated with 8-hydroxyquinoline (or with o-phenanthroline) (Bedard et al., 1984), and efficient conjugation in turn requires gene induction (Trueheart et al., 1987). Therefore induction of the general control response is unrelated to the inhibition of pre-rRNA transcription or cleavage. These findings also show that during conjugation there is little need for continued rRNA production.

In addition to the RNA effects, most of the inhibitors tested also caused ATP pool perturbations (Fig. 2) that were similar to the effects on pyrimidine triphosphate pool specific activity noted earlier for some of the inhibitors (Singer and Johnston, 1979; Li et al., 1984). The decrease in ATP pool specific activity cannot easily be ascribed to expansion of ATP pools from turnover of stable RNA (Johnston and Singer, 1978); therefore some of these inhibitors may also release sequestered nucleotides or inhibit uptake of exogenous precursors. Whether these postulated effects influence transcription in the uracil auxotrophic cells used in these experiments remains to be determined. However, ATP pool perturbations do not always accompany start arrest (see Fig. 2H), and start inhibition is unrelated to purine or pyrimidine auxotrophy; both of these observations suggest that nucleoside triphosphate pool perturbations are not involved in the start arrest caused by these inhibitors.

The biosynthetic effects of start inhibitors were assessed here at concentrations that produce start inhibition rather than at concentrations optimized for biosynthetic effects. It remains to be seen if other inhibitor concentrations cause more selective biosynthetic inhibition. It is striking, therefore, that of the parameters assayed for these inhibitors of start only the inhibition of pre-rRNA transcription could be implicated in start inhibition.

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3 L. M. Veinot-Drebot, R. A. Singer, and G. C. Johnston, unpublished observations.
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Even though most of the inhibitors studied here inhibited both pre-rRNA transcription and cleavage, it is unlikely that the inhibitors act through similar mechanisms. This conclusion is illustrated by a comparison of the effects of two of the inhibitors studied here, l-ethionine and sinfungin, with the similar effects of heat shock. Upon heat shock, the inhibition of cleavage leading to accumulation of 35 S pre-rRNA (Veinot-Drebot et al., 1988) accompanies the decreased production of ribosomal proteins (Kim and Warner, 1983). The absence of some of these ribosomal proteins probably inhibits the initial cleavage reaction. In contrast, the two start inhibitors affect the RNA substrate for cleavage, which is methylated pre-rRNA. Both l-ethionine and sinfungin cause undermethylation of rRNA (Singer et al., 1978; Li et al., 1985), indicating that the methylation of pre-rRNA (Udem and Warner, 1972; Klootwijk and Planta, 1973) is decreased by these inhibitors. The accumulation of 35 S pre-rRNA molecules caused by l-ethionine and sinfungin (Fig. 3A) suggests that these undermethylated pre-rRNA molecules may be inefficient substrates for cleavage even in the presence of ribosomal proteins. Therefore these observations suggest that at least with respect to the initial cleavage of pre-rRNA, the start inhibitors l-ethionine and sinfungin work through a different mechanism than heat shock to yield the same general phenotypes.

The quantitative features of the dual inhibitions of pre-rRNA transcription and of cleavage also suggest that the inhibitors studied here affect rRNA synthesis through more than one mechanism. For example, both o-phenanthroline and 8-hydroxyquinoline (Singer and Pringle, 1983) inhibit transcription to roughly the same extent as cleavage and in comparison with the effects of l-ethionine and sinfungin must affect rRNA production differently.

The inhibition of start that is produced by o-phenanthroline and by 8-hydroxyquinoline is abrogated by zinc ions (Johnston and Singer, 1978), in keeping with the known actions of these inhibitors as zinc chelators. Although it has been shown that certain metal chelates of o-phenanthroline can cause single-strand nicks in DNA (Marshall et al., 1981), this DNA-nicking activity is not a consideration in this in vivo situation because DNA damage in yeast brings about arrest in G2 (Brunborg and Williamson, 1978; Jordan and Laskowski, 1987) rather than the start arrest seen here. Furthermore, the ability of ferrous ions as well as zinc ions to abrogate inhibition by o-phenanthroline and 8-hydroxyquinoline (Singer and Johnston, 1982) suggests that in chelated configuration these inhibitors are inactive. All these observations suggest that the inhibition caused by o-phenanthroline and 8-hydroxyquinoline may be due to chelation of zinc ions, perhaps the zinc ions found in yeast RNA polymerase I (Auld et al., 1976; Memet et al., 1988) or in transcription factors (Evans and Hollenberg, 1988).
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