The effects of inhibitors of protein synthesis upon transcription have been re-examined. Cycloheximide (1 μg/ml) inhibits incorporation of uridine into RNA of P1798.S20 lymphosarcoma cells. Filter hybridization studies indicate that labeling of pre-rRNA is inhibited 60–80% after 1 h and quantitative S1 nuclease mapping reveals a corresponding decrease in the amount of cellular pre-rRNA. Cycloheximide also inhibits labeling of 5 S RNA and tRNA, but incorporation of uridine into poly(A+) RNA is unaffected. Transcription experiments carried out in nuclei from cycloheximide-treated cells indicate that the inhibitor causes a selective decrease in the activity of RNA polymerases I and III.

Cell-free extracts from P1798.S20 were used to transcribe the cloned mouse rRNA gene, Syrian hamster 5 S RNA gene, and the Drosophila tRNA\(^{\text{Arg}}\) gene. Extracts from cycloheximide-treated cells were inhibited in this respect. Transcription of rRNA and 5 S RNA genes was inhibited by 90% after 2 h and 50% inhibition occurred within 20–30 min. Transcription of the tRNA gene was inhibited 75% after 2 h with a half-time of ~1 h. Inhibition was due neither to a direct effect of cycloheximide nor to the presence of nucleases or diffusible inhibitors of transcription. Moreover, transcription of rDNA in extracts from cycloheximide-treated cells could be restored by the addition of a partially purified initiation factor preparation. The data indicate that inhibition of protein synthesis results in rapid depletion of transcription factors that are required for initiation by RNA polymerases I and III. Among these is the glucocorticoid-regulated rDNA initiation factor designated TFIC.

Inhibition of protein synthesis in yeast is accompanied by inhibition of pre-rRNA synthesis (Foury and Goffeau, 1973; Gross and Pogo, 1976, a and b). The synthesis of pre-rRNA in mammalian cell culture lines is inhibited by amino acid starvation (Franze-Fernandez and Pogo, 1971; Grummt and Grummt, 1976) as well as inhibition of protein synthesis by cycloheximide and puromycin (Warner et al., 1966; Soeiro et al., 1968; Craig and Perry, 1970; Chesterton et al., 1975; Mishima et al., 1979; see also Warner, 1974; Hadjilov and Nikolaev, 1976). Injection of cycloheximide into rats has been reported to cause rapid inhibition of rRNA synthesis in liver and liver nucleoli (Yu and Feigelson, 1972), although these observations have been challenged (Stoyanova and Dabeva, 1980). These data have been interpreted to indicate the existence of an rDNA transcription factor that exhibits a very short biological half-life.

In this laboratory, we have begun to study hormonal regulation of transcription of rDNA and have identified a hormone-regulated transcription initiation factor (Cavanaugh et al., 1984). Kinetic studies suggest that this protein turns over rapidly and may be a logical candidate to account for the effects of protein synthesis inhibitors on transcription of rDNA (Yu and Feigelson, 1973). Other studies suggest that the rate of transcription of the 5 S RNA gene may be regulated by partitioning of transcription initiation factor IIIA between the gene and nascent 5 S RNA (Pelham and Brown, 1980; Honda and Roeder, 1980; Hanas et al., 1983). In the absence of ribosome assembly, 5 S RNA may accumulate to the extent that transcription of the gene is inhibited as the factor is diverted to form the TFIIIA-5 S RNA complex. If this mechanism is correct, inhibition of translation should result in rapid inhibition of synthesis of 5 S RNA as TFIIA is depleted. Similar factors may play a role in transcription of tRNA and rRNA genes and could provide a general mechanism of coordination of synthesis of ribosomal components. To test this hypothesis, we have re-examined the effects of cycloheximide upon RNA synthesis in lymphosarcoma P1798 cells in culture.

The data demonstrate that synthesis of pre-rRNA, 5 S RNA, and tRNA is rapidly inhibited after cessation of protein synthesis and suggest that transcription of class I and III genes requires a protein(s) of short biological half-life.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

*Effect of Cycloheximide upon RNA Synthesis in Cells—Preliminary experiments were undertaken to determine the optimum concentration of cycloheximide for inhibition of protein synthesis in P1798 cells. Incorporation of [\(^{3}H\)]leucine into trichloroacetic acid-precipitable material was inhibited 90–95% in the presence of 1 μg/ml cycloheximide. Higher concentrations produced no additional effect and maximum inhibition was achieved within 1–2 h. The effects of cycloheximide upon incorporation of [\(^{3}H\)]uridine are shown in Fig. 1. Total uridine incorporation during a 15-min pulse-labeling period (diamonds in Fig. 1) was inhibited approximately 80%*

1 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-5048; cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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in this experiment and maximum inhibition was observed within 2–4 h after the addition of cycloheximide. RNA was extracted and the amount of labeled pre-rRNA was measured by hybridization to filter-immobilized pI23 (open circles). As shown in Fig. 1, incorporation of [3H]uridine into pre-rRNA (open circles) paralleled inhibition of total [3H]uridine incorporation. Quantitative S1 nuclease mapping was used to estimate the amount of pre-rRNA in cycloheximide-treated cells. The observed decrease in the amount of cellular pre-rRNA was paralleled by the inhibitory effects of cycloheximide upon synthesis of labeled pre-rRNA. These observations are consistent with the very short half-life (5–10 min) of the rRNA primary transcript (Perry, 1978; Gurney, 1985). In a brief pulse-labeling experiment of this sort, one would expect this species of RNA to be disproportionately labeled. The rapid turnover likewise accounts for the rapid disappearance of the primary transcript following inhibition of synthesis.

Cultures of P1798 were treated with cycloheximide for various periods of time, the cells were pulse-labeled with [3H]uridine, and labeled RNA species were resolved as described under “Experimental Procedures.” As shown in Fig. 2, pulse-labeling of pre-rRNA was inhibited ~60%. Labeling of 5 S RNA and tRNA was reduced by 40–50%; there was no detectable decrease in the amount of 5 S RNA or tRNA, as estimated by the intensity of staining with ethidium bromide. Inhibition of labeling of 5 S RNA was also confirmed by filter hybridization to pTH1 (data not shown). Labeling of poly(A+)-labeled pre-rRNA. These observations are consistent with the lack of effect upon labeling of poly(A+) RNA. RNA polymerase activity was also measured in extracts from control and cycloheximide-treated cells (Table IB). Exposure to cycloheximide (1 μg/ml, 2 h) did not affect transcription of calf thymus DNA by RNA polymerases I or II. The data indicate that cycloheximide causes inhibition of transcription by RNA polymerases I and III. This was confirmed by measuring the activity of these enzymes in nuclei isolated from control and cycloheximide-treated cells, as shown in Table IA. The assays were carried out in the presence of exogenous template and under these conditions one measure, primarily elongation of RNA chains that were initiated prior to isolation of nuclei. Nuclei from cycloheximide-treated cells exhibited a significant decrease in the activity attributable to RNA polymerases I and III. No significant decrease was observed in RNA polymerase II activity, consistent with the lack of effect upon labeling of poly(A+) RNA. RNA polymerase activity was also measured in extracts from control and cycloheximide-treated cells (Table IB). Exposure to cycloheximide (1 μg/ml, 2 h) did not affect transcription of calf thymus DNA by RNA polymerases I or II. The data indicate that neither nonspecific initiation nor nucleotidyltransferase activity was inhibited and suggest that cycloheximide acts at the level of specific initiation upon class I and class III genes.

**Effect of Cycloheximide or Emetine upon RNA Synthesis in Vitro—**S100 extracts were prepared and assayed for the ability to transcribe cloned mouse rDNA (pPMAB/pPuuII), the Syrian hamster 5 S RNA gene (pTH1), and the Drosophila tRNA^H^ gene (pYH8). The results are shown in Fig. 3. Transcription of the PuuII-truncated, cloned mouse rDNA RNA was unaffected by a 2-h exposure to cycloheximide indicating that the apparent inhibition of synthesis of other RNA species is not a reflection of decreased specific activity of intracellular nucleotide pools.

Transcription of rRNA, 5 S RNA, and tRNA Genes
Table I
RNA polymerase activity

A. In isolated nuclei

| Polymerase | Control (Relative) | Cycloheximide (Relative) |
|------------|--------------------|--------------------------|
| Pol I      | 106 (3.6)          | 35 (1.5)                 |
| Pol II     | 29 (1)             | 23 (1)                   |
| Pol III    | 8 (0.3)            | 1 (0.04)                 |
| Total      | 143 (5)            | 59 (2.6)                 |

B. In S100 extracts

| Polymerase | Cpm/10⁶ nuclei |
|------------|----------------|
| Pol I      | 1500           |
| Pol II     | 1370           |
| Pol III    | 180            |

Fig. 3. Transcription in vitro. S100 extracts were prepared from 1 liter cultures of control cells (lanes a) and cells treated for 2 h with 1 μg/ml cycloheximide (lanes b) or 1 μM emetine (lanes c). Extracts were used to transcribe the cloned genes containing the rRNA promoter (prMAB/PouII), the 5 S RNA gene (pTH1), and the tRNA<sup>Ar</sup> gene (pYH48). The products were resolved on polyacrylamide gels as described and the arrows indicated the calculated positions of RNAs of the following sizes: rRNA, 292 nt; 5 S RNA, 122 nt; tRNA, 75 and 100 nt.

The abbreviations used are: nt, length in nucleotides of single-stranded nucleic acids; kb, kilobase pairs of double-stranded nucleic acids; hp, base pairs of double-stranded nucleic acids; poly(A<sup>+</sup>) RNA, presumptive mRNA that contains (3') oligoadenylate tails of sufficient length to form stable hybrids with oligo(dT)-cellulose under the stated conditions; SDS, sodium dodecyl sulfate; TFIC, glucocorticoid-regulated rDNA initiation factor; TFID, stable complex factor(s) that elute from phosphocellulose in 1 M KCl.

Fig. 4. Time course of cycloheximide inhibition. S100 extracts were prepared from 10⁶ cells that had been treated with 1 μg/ml cycloheximide for various periods of time. These extracts were used to transcribe the cloned genes encoding pre-rRNA (prMAB, open circles), 5 S RNA (pTH1, closed circles), or tRNA<sup>Ar</sup> (pYH48, closed diamonds). The products were resolved by electrophoresis on polyacrylamide gels and specific transcripts were detected by autoradiography, excised from the gels, and counted for 32P by liquid scintillation.

Transcription of the cloned 5 S RNA gene results in formation of a 122-nt RNA (5 S RNA, lane a), whereas transcription of the tRNA<sup>Ar</sup> gene yields a family of transcripts and processing intermediates of 75–100 nt in length (tRNA, lane a). The ability to transcribe 5 S RNA and tRNA genes was reduced in S100 extracts from cycloheximide-treated or emetine-treated cells (lanes b and c, respectively). The time course of inhibition was determined by preparing S100 extracts from cells treated with cycloheximide for various intervals. As shown in Fig. 4, transcription of the cloned rRNA and 5 S RNA genes was inhibited ~90% within 2 h; 50% inhibition occurred approximately 20 min after addition of cycloheximide. Transcription of the cloned tRNA gene was reduced by ~75% under these conditions and 50% inhibition occurred 50–60 min after addition of the inhibitor.

Inhibition of transcription in vitro was not due to a direct effect of cycloheximide upon the enzymes and/or initiation factors. As shown in Fig. 5, addition of cycloheximide to S100 extracts did not inhibit transcription of the genes encoding rRNA, 5 S RNA, or tRNA. As an additional control, a mixing experiment was carried out as shown in Fig. 6. Mixtures of S100 extracts from control and cycloheximide-treated cells were able to transcribe the cloned genes, indicating that inhibition of transcription was not due to the presence of nucleases or diffusible inhibitors in extracts from cycloheximide-treated cells.

Reconstitution of Transcription in Vitro—An S100 extract was fractionated by chromatography on DEAE-cellulose and phosphocellulose as described under "Experimental Procedures." Four fractions, designated A, B, C, and D, were obtained and none of these was capable of specific transcription of mouse rDNA (Fig. 7, lanes a–d). Fraction C from the phosphocellulose column (eluted at 600 mM KCl) was capable of reconstituting transcription of rDNA in extracts from cycloheximide-treated cells (Fig. 7, lane g).

Phosphocellulose fraction C contains at least two components of the rDNA transcription complex: a small but detectable amount of RNA polymerase I and the hormone-regulated initiation factor TFIC (Cavanaugh and Thompson, 1985). Either or both of these factors could account for the effects of cycloheximide. A template exclusion protocol was employed to determine if RNA polymerase I from cycloheximide-treated cells was capable of specific initiation. The rationale for such
Transcription of rRNA, 5S RNA, and tRNA Genes

FIG. 5. The addition of cycloheximide to S100 extracts from control cells. The appropriate cloned genes, as described in the legend to Fig. 3, were transcribed in vitro in the presence or absence of cycloheximide. Lanes a, c, and e are control reactions whereas lanes b, d, and f contain the products of transcription reactions carried out in the presence of 2 μg/ml cycloheximide.

Fig. 6. Mixing of extracts from control and cycloheximide-treated cells. The cloned genes were transcribed with 5 μl of control S100 (cont), 5 μl of S190 from cells treated 2 h with 1 μg/ml cycloheximide (cyclo), or a mixture of 5 μl of each. The positions of the appropriate transcripts are indicated by arrows.

a procedure is as follows. Extracts were preincubated with template under conditions that permitted formation of stable, preinitiation complexes. Formation of such complexes was evidenced by the observation that preincubation with a given template precluded transcription of a second template added subsequently. An extract from control cells was heated for 15 min at 45°C to inactivate RNA polymerase I. Such extracts have been previously shown to be incapable of transcription but may be reconstituted by the addition of partially purified or highly purified RNA polymerase I (Cavanaugh and Thompson, 1984). The heat-inactivated extract was preincubated with prMAB truncated with SmaI (prMAB/SmaI) so as to introduce a double-stranded break at +155 bp. An extract from cycloheximide-treated cells was preincubated with prMAB truncated with PvuII at +292 bp (prMAB/PvuII). After preincubulation, the extracts were mixed and prMAT11/PvuII was added. This third template was truncated with PvuII at +350 bp. Transcription was carried out and the products were characterized as shown in Fig. 8, lanes a–c. The heated extract was incapable of transcription (lane a) and transcriptional activity was reduced in the cycloheximide-treated extract (lane b). When the two reactions were mixed, however, both of the preincubated templates were transcribed to yield RNAs of 292 nt (prMAB/PvuII) and 155 nt (prMAB/SmaI). The third template (prMAT11/PvuII) was not transcribed. These data suggest that both heated and cycloheximide-treated extracts are capable of forming stable, preinitiation complexes, thereby excluding transcription of templates added subsequent to preincubation. Preinitiation complexes formed in heat-treated extracts cannot support transcription because they do not contain functional RNA polymerase I. However, RNA polymerase I from cycloheximide-treated cells can reconstitute heat-treated extracts indicating that cycloheximide does not cause inactivation of the enzyme. Moreover, preinitiation complexes form in extracts from cycloheximide-treated cells and can support transcription if supplemented with one or more heat-stable factors from control extracts.

Extracts from dexamethasone-treated cells contain functional RNA polymerase I but are depleted of the transcription initiation factor TFIC (Cavanaugh and Thompson, 1983, 1985). Mixing experiments indicate that extracts from hormone-treated cells do not reconstitute extracts from cycloheximide-treated cells (Fig. 8, lanes d, e, and f). Therefore, at least one factor in common is involved in both cycloheximide- and glucocorticoid-mediated inhibition of transcription of rDNA. Finally, partially purified TFIC is capable of reconstituting extracts from cycloheximide-treated cells (Fig. 8, lanes g and h). In this experiment, TFIC was preincubated with prMAB/SmaI and a cycloheximide-treated extract was preincubated with prMAB/PvuII. TFIC does not carry out specific transcription of rDNA (lane g); the high level of nonspecific transcription is due to contamination of this fraction with RNA polymerase I. When the reactions were mixed, only prMAB/PvuII was transcribed (lane h). These data confirm
The treated extract was preincubated with prMAB/PuuII. Thereafter, the under "Experimental Procedures." In parallel, a cycloheximide-treated extract was preincubated with prMAB/SmaI as described earlier. 

Lane d, an extract from P1798 cells, treated 24 h with 0.1 μM dexamethasone, transcribing prMAB/SmaI. Lane e, an extract from cycloheximide-treated cells transcribing prMAB/PuuII. Lane f, a mixing experiment in which a hormone-treated extract was preincubated with prMAB/PuuII and mixed with a cycloheximide-treated extract that had been preincubated with prMAB/PuuII. Lane g, 5 μl of TFIC (0.5 μg of protein) transcribing prMAB/SmaI. Lane h, a cycloheximide-treated extract was preincubated with prMAB/PuuII and mixed with a similar reaction in which 5 μl of TFIC had been preincubated with prMAB/PuuII. Transcription was initiated by the addition of nucleoside triphosphates and the products were resolved as described. Lane m contains 5' end-labeled fragments derived by digestion of 6x174 with HaeIII. The smallest fragment shown in lane m has a size of 185 nt.

the observation that extracts from cycloheximide-treated cells are capable of forming preinitiation complexes but are depleted of TFIC.

**DISCUSSION**

Cycloheximide inhibits the synthesis of pre-rRNA, 5 S RNA, and tRNA in P1788 cells. The effect prevails in nuclei and in S100 extracts from cycloheximide-treated cells indicating that inhibition occurs at the level of transcription. Three observations suggest that the effects of cycloheximide are mediated via inhibition of translation rather than by a direct effect upon transcription; (i) inhibition in culture is specific for RNA polymerases I and III, indicating that cycloheximide is not a nonspecific inhibitor of transcription; (ii) cycloheximide does not inhibit transcription in vitro; (iii) the effects of cycloheximide are identical with those of emetine, a structurally dissimilar inhibitor of translation. Moreover, transcription of rDNA in extracts from cycloheximide-treated cells can be reconstituted by the addition of partially purified transcription factors from control cells. This indicates that the transcriptional defect exhibited by these extracts is not due to general degradation or denaturation.

It has been reported that transcription of rDNA is influenced by intracellular nucleotide pools (Grummt and Grimmt, 1976). Cycloheximide may alter pool sizes under certain circumstances (Stoyanova and Dabeva, 1980). However, labeling of poly(A+) RNA was not reduced in cycloheximide-treated cells suggesting that changes in UTP pools do not occur under the experimental conditions employed. Mishima et al. (1979) have observed that cellular ATP and GTP pools are unaffected by treatment with 100 μg/ml cycloheximide for 90 min. Although we have not measured nucleotide pool sizes, we assume that pool sizes remain relatively constant and do not account for inhibition of transcription under the circumstances described in this report.

Our working hypothesis proposes that initiation of transcription by RNA polymerases I and III requires the continual synthesis of factors that are rapidly degraded or consumed during the transcription process. Attempts to reconstitute transcription of 5 S RNA and tRNA genes have yielded equivocal results in that more than one phosphocellulose fraction contains stimulatory activity. Our primary interest is in transcription of rDNA and the mechanism of action of cycloheximide upon RNA polymerase III has not been pursued.

Inhibition of rRNA synthesis results from a decrease in the amount or activity of a protein that co-purifies with the glucocorticoid-regulated rDNA transcription initiation factor TFIC. The data strongly suggest that TFIC exhibits a short biological half-life and is rapidly depleted after inhibition of translation. This is consistent with an early report that indicated that a protein of short half-life is involved in glucocorticoid regulation of rRNA synthesis in rat liver (Yu and Feigelson, 1972, 1973). Other data suggest that TFIC may be involved in inhibition of transcription of rDNA in serum or amino acid-starved cells (Grimmt, 1982). This protein therefore appears to play a central role in regulating the rate of synthesis of the precursor for rRNAs. In order to prove this hypothesis, it will be necessary to purify TFIC.

To date, none of the ancillary RNA polymerase I transcription factors has been purified to homogeneity and the biochemical properties of the rDNA transcription complex are not completely elucidated. Fractionation studies suggest that at least three classes of proteins are involved: RNA polymerase I; the stable complex factor(s) that elute from phosphocellulose in 1 M KCl (TFID); and the initiation factor TFIC (Mishima et al., 1982). Poly(ADP-ribose) polymerase may also be involved (Kurl and Jacob, 1985). Template exclusion experiments indicate that extracts from cycloheximide-treated cells are capable of forming stable preinitiation complexes. It is therefore unlikely that the effects of cycloheximide are attributable to the stable complex factors designated TFID. TFIC is rather strongly associated with the polymerase and, in our experience, it is difficult to completely resolve TFIC from the enzyme. The most highly purified preparations of TFIC contain <1% of the polymerase I activity that was present in the S100 extracts and it is difficult to preclude the possibility that TFIC represents a minor subpopulation of functional RNA polymerase I. Reconstitution studies with heat-inactivated extracts argue against this possibility. The polymerase from cycloheximide-treated extracts is functional when mixed with heated extracts from control cells. Conversely, a heat-stable factor from control cells can reconstitute cycloheximide-treated extracts. Since heat-treated extracts contain no detectable RNA polymerase activity, it is difficult to argue that reconstitution of transcription under these circumstances is attributable to the polymerase. The simplest interpretation of these data is that TFIC is distinct from RNA polymerase I. However, one may not preclude the possibility that TFIC is an exchangeable polymerase subunit or a heat stable enzyme that modifies the polymerase in some way so as to facilitate specific initiation.

Elucidation of the mechanism that underlies the effect of
inhibitors of translation requires purification and characterization of the factors involved. At the present time, the number of factors involved is unknown. We are intrigued by the possibility that a class of proteins of short biological half-life may be involved in coordinating the rate of translation and the rates of synthesis of rRNAs, 5S RNAs, and tRNAs. Such a mechanism could have physiological significance in preventing overproduction of these RNA species under conditions in which ribosome assembly is limited by the availability of the ribosomal proteins and may be analogous to a stringent control mechanism in mammalian cells.

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