Inhibition of Nucleoplasmic Transcription and the Translation of Rapidly Labeled Nuclear Proteins by Low Concentrations of Actinomycin D in Vivo. Proposed Role of Messenger RNA in Ribosomal RNA Transcription†

Thomas J. Lindell,* Anthony F. O'Malley, and Brian Puglisi

ABSTRACT: Initial observations in isolated rat liver nuclei and nucleoli suggested that actinomycin D does not inhibit nucleolar transcription in vitro in the same concentration range effective for the inhibition of rRNA synthesis in vivo (Lindell, T. J. (1976) *Nature (London) 263,* 347–350). These observations have been extended in vivo to demonstrate that low concentrations of actinomycin D (0.001–0.1 μg), administered on a μg/g basis, cause a partial inhibition of nucleoplasmic RNA polymerase II as assayed in isolated liver nuclei. In cells in culture, actinomycin D at 0.1 μg/mL caused a partial inhibition of RNA polymerase II with a concomitant marked time dependent effect on nucleolar RNA polymerase I activity ($t_{1/2}$ = 20 min). This same concentration range of actinomycin D in vivo also inhibits the synthesis of rapidly labeled nuclear proteins depending on the concentration of actinomycin D administered. At a concentration of 0.5 μg/g of body weight, actinomycin D is observed to cause a "superinduction" of the synthesis of rapidly labeled nuclear proteins of rat liver. These observations suggest that rRNA transcription may be under the control of mRNA transcription and that the synthesis of these mRNAs is inhibited by low concentrations of actinomycin D rather than an inhibition of nucleolar transcription directly. To account for the rapid onset of action of actinomycin D in the inhibition of rRNA synthesis, it is suggested that these mRNAs have a very rapid turnover.

Prior to knowledge of the mechanism of action of α-amanitin Fiume & Laschi (1965) described that administration of this toxin to KB cells in culture rapidly caused a concentration dependent fragmentation of nucleoli. Subsequent studies demonstrated that the Mn^{2+}, high ammonium sulfate stimulated RNA polymerase activity is inhibited by this toxin (Stirpe & Fiume, 1967). α-Amanitin was ultimately shown to specifically inhibit nucleoplasmic DNA-dependent RNA polymerase II in a variety of eukaryotic sources (Lindell et al., 1970; Kedinger et al., 1970; Jacob et al., 1970a). More recent studies have demonstrated that higher concentrations of α-amanitin also inhibit RNA polymerase III which is also found in the nucleoplasm (Weinmann & Roeder, 1974).

The results of Fiume & Laschi (cited above) pose a paradoxical question as to how a specific inhibitor of nucleoplasmic RNA polymerase II could affect nucleolar structure (and function) since nucleolar integrity is closely related to the synthesis of ribosomal RNA (rRNA) (Smetana & busch, 1970). Two groups subsequently reported effects of α-amanitin administered in vivo to rats on the synthesis of liver messenger (mRNA) and rRNA (Jacob et al., 1970b; Tata et al., 1972). Two other groups also studied the effect of α-amanitin in vivo on the integrity of liver nucleoli after administration of α-amanitin to intact animals (Marinozzi & Fiume, 1971; Petrov & Sekeris, 1971). The former two studies (Jacob et al., 1970b; Tata et al., 1972) suggested a potential role of mRNA transcription in the maintenance of rRNA transcription in vivo. Since the inhibition of rRNA transcription was only transient, these proposals have not been widely acknowledged. The reason for the lack of effect of α-amanitin in vivo is apparently due to the inability of this peptide toxin to penetrate the cell membrane in all cells (Boctor & Grossman, 1973).

In light of the above model suggesting a role of nucleoplasmic mRNA transcription in the control of rRNA transcription, the effect of actinomycin D on transcription in isolated rat liver nuclei in vitro was investigated. It was found that actinomycin D did not inhibit α-amanitin insensitive RNA polymerases I plus III in nuclei or RNA polymerase I in isolated nucleoli in the concentration range 0.001–0.1 μg/mL (Lindell, 1976). This is the same concentration range which was found to be effective in the inhibition of rRNA synthesis in cells in culture (Perry, 1963; Perry & Kelley, 1970). It was found, however, that actinomycin D inhibited a fraction (20%) of nucleoplasmic transcription (RNA polymerase II) in isolated nuclei and this inhibition was dependent upon the concentration of actinomycin D employed (Lindell, 1976). These observations predict that actinomycin D has a higher affinity for specific nucleoplasmic loci than for rDNA.

The studies contained in this report were designed to ask whether there is a detectable inhibition of nucleoplasmic RNA polymerase II in assays of nuclei isolated from tissues or cells treated with low concentrations of actinomycin D. Further, if actinomycin D in vivo selectively inhibits the synthesis of certain unique mRNAs, can this purported mechanism of action account for the rapid inhibition of rRNA transcription? An inhibition of RNA polymerase II has been found after the in vivo administration of actinomycin D which is also manifest in an inhibition of the synthesis of rapidly labeled proteins which migrate to the nucleus. These results can be interpreted to involve the selective inhibition of certain mRNAs from which these proteins are translated. For actinomycin D to have an extranucleolar mechanism of action, the mRNAs for these proteins must have very rapid turnovers.

† From the Department of Pharmacology, Arizona Health Sciences Center, Tucson, Arizona 85724. *Received October 11, 1977.* Supported by National Institutes of Health Grants GM-18764 and GM-22897, American Cancer Society (Arizona Section) and a Basic Research Support Grant to the University of Arizona Health Sciences Center to T. J. Lindell. A. F. O'Malley was the recipient of a National Cancer Institute Postdoctoral Fellowship (CA-05363).

0006-2960/78/0417-1154$01.00/0

Materials and Methods

Animals. Rats employed were 10–14 day old, Sprague-Dawley (male and female), preweaned pups (littermates weighing 23–30 g), and were used immediately after removal from the dame. The reasons for using small rats were twofold: first, the animals are small enough to allow for rapid distribution of drugs and/or labeled amino acids; and second, because the liver is large enough (0.4 g) to allow for the isolation of sufficient nuclei for multiple RNA polymerase and DNA assays. Animals were injected intraperitoneally with actinomycin D (Merck) or cycloheximide (Sigma) as indicated. Fifteen minutes after the administration of the inhibitor, ^{14}C- or ^{3}H-labeled amino acid mixture (New England Nuclear) was administered intraperitoneally. Labeled amino acid mixtures were neutralized with 0.1 N NaOH prior to injection. All injections were given in volumes no greater than 0.10 mL.

Cell Culture. Primary chick fibroblasts were isolated and grown as described by Dinowitz et al. (1977). Confluent primary cultures were treated with 0.05% trypsin (Gibco) and diluted 1:2. When the secondary cultures were confluent, they were treated with trypsin as above and split into three 2-L sealed roller bottles. After the cells had grown for 24 h, two bottles were treated with 0.1 μg/mL actinomycin D, while the other served as control. Cells were treated with actinomycin D for 10 and 20 min, respectively. All subsequent steps for cell disruption were carried out in ice water.

Cell Fractionation. Cells were disrupted by the method of Borun et al. (1967) except that Triton X-100 (Sigma) was used instead of Nonidet NP-40. The cells were washed with Tris-buffered saline (0.14 M NaCl, 0.05 M KCl, 0.007 M Na_2HPO_4, 0.005 M glucose, 1 mM phenylmethanesulfonyl fluoride (Sigma), and 0.02 M Tris, pH 7.4) after which RSB (0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris, pH 7.4, containing 1 mM phenylmethanesulfonyl fluoride and 0.5% Triton X-100) was added for 5 min. The cells, with disrupted cytoplasmic membranes, were removed with the aid of a rubber policeman, vortexed, and centrifuged at 1500*g*. Nuclear pellets, for the assay of RNA polymerase activities, were suspended in 2 mL of 1 M sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 0.05 M Tris-HCl, pH 7.5, by hand with a glass–Teflon homogenizer.

Isolation of Liver Nuclei. Nuclei were isolated by the method of Blobel & Potter (1966) as modified below. At appropriate times, rat pups were killed by decapitation with a scissors; livers were removed (0.4 g each) and placed in 1.0 mL of 0.25 M sucrose (Mann), 50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$ (pH 7.9) (buffer A). All buffers contained 1 mM phenylmethanesulfonyl fluoride which was added just prior to use of the buffers by dilution of a 0.25 M solution of phenylmethanesulfonyl fluoride (in ethanol) (Lindell, 1975). Livers were homogenized in a glass–Teflon Potter-Elvehjem homogenizer (A. H. Thomas) with a motor-driven pestle ten times. The volume was measured and 2 volumes of 2.3 M sucrose in buffer A added and mixed well. Individual nuclear homogenates were placed in cellulose nitrate tubes, underlaid with 1 mL of 1.9 M sucrose in buffer A, and centrifuged in a Beckman SW 50.1 rotor at 25 000 rpm (70 000*g*) for 20 min at 4 °C. Individual nuclear samples were then gently washed with 0.25 M sucrose in buffer A to remove a layer of fibrous material. Nuclei were then resuspended by gentle hand homogenization as above in 1.2 mL of 1 M sucrose containing 5 mM $MgCl_2$, 1 mM dithiothreitol (Sigma), and 0.05 M Tris-HCl, pH 7.5.

Assay of Nuclear RNA Polymerases. RNA polymerase assays in rat liver nuclei were performed as described by Zerwekh et al. (1974) using a single concentration of $(NH_4)_2SO_4$ (0.05 M) rather than two concentrations of this salt. Individual nuclear assays (five in the presence and five in the absence) of α-amanitin (0.1 μg/assay) were incubated at 30 °C for 5 min. Some assays contained α-amanitin at 400 μg/mL to inhibit RNA polymerase III (Weinmann & Roeder, 1974). All incubations were terminated by pipetting 100-μL aliquots of each assay onto Whatman DE-81 filter discs which were immediately placed in 5% Na_2HPO_4, washed, and counted as described by Lindell et al. (1970). Nucleoplasmic form II RNA polymerase activity was quantitated by subtracting the cpm incorporated at 0.05 M ammonium sulfate in the presence of α-amanitin, from those performed at the same ammonium sulfate concentration in the absence of α-amanitin. Nuclear forms I and III RNA polymerase activities were quantitated by subtracting the cpm obtained from control incubated samples containing 80 μg/mL actinomycin D plus α-amanitin (0.1 μg/assay) from the cpm incorporated in the presence of α-amanitin alone (Lindell, 1976). There is no linear incorporation of [^{3}H]UMP into RNA in this control. This control is necessary as a background subtraction since a small but significant number of counts (150–250 cpm) are found even though no linear incorporation has taken place.

Nuclei from chick fibroblasts were assayed in an identical manner except the amount of unlabeled UTP was 0.00125 μmol per assay with 2 μCi of [^{3}H]UTP.

Activities of RNA polymerases are expressed as picomoles of UMP incorporated per 5 min per mg of DNA. DNA determinations were done on identical 50-μL aliquots of the same nuclear samples used for RNA polymerase assays. DNA determinations were done in triplicate by the method of Burton (1956) using type I calf thymus DNA (Sigma) as a standard.

Pulse Labeling of Nuclear Proteins. Rapidly labeled nuclear proteins of rat liver are characterized as those which are found in the nucleus within 15 min after a pulse with a ^{14}C- or ^{3}H-labeled amino acid mixture administered intraperitoneally. When livers were removed from the rats, they were washed with about 10 mL of 0.25 M sucrose in buffer A to remove any free unincorporated amino acids. Nuclei which had been labeled with amino acids were isolated and suspended as described above. Fifty microliters was pipetted onto Whatman 3 MM filters (2.3 cm), washed with cold 5% trichloroacetic acid (three times at 4 mL/filter), twice with 95% ethanol, and twice with diethyl ether, and counted as above. All incorporation data are expressed relative to the amount of nuclear DNA as determined above on triplicate 50-μL aliquots of nuclei. All scintillation counting was performed on either a Searle Isocap/300 or Mark III.

Cytoplasmic protein (supernate from the centrifugation of individual nuclear homogenates) was applied to 3 MM filters (100 μL), washed, and counted as described for nuclei above. Protein determinations of cytoplasm were performed on trichloroacetic acid precipitated protein by the method of Lowry et al. (1951).

Significance of experimental results was measured using the Student's *t* test for unpaired samples.

Results

Effect of Actinomycin D in Vivo on the Activity of Nuclear RNA Polymerases in Vitro. Since it appeared from in vivo studies with α-amanitin that this peptide toxin was probably not getting into cells effectively (Jacob et al., 1970b; Tata et al., 1972), we have examined the effect of actinomycin D in

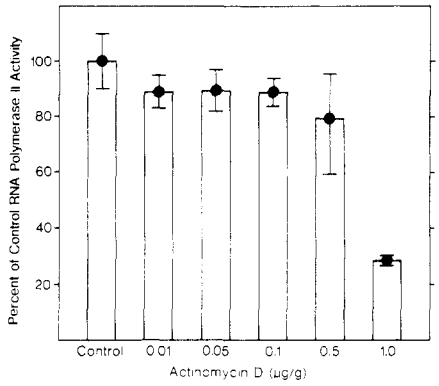

FIGURE 1: Effect of actinomycin D in vivo (rat pups) on liver nuclear RNA polymerase II assayed in vitro. Actinomycin D was injected intraperitoneally into rat pups at the various concentrations indicated (μg/g of body weight). Liver nuclei were isolated 1 h later and nuclear RNA polymerases assayed as described in Materials and Methods. Each point represents the average of three individual liver nuclear samples ± SEM nucleoplasmic RNA polymerase II activity. Control activity was 231 pmol of UMP incorporated per 5 min per mg of DNA.

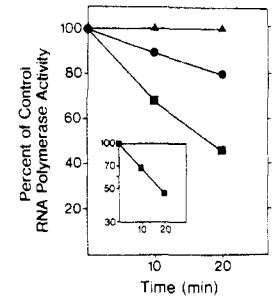

FIGURE 2: Effect of actinomycin D at 0.1 μg/mL on RNA polymerase activities in nuclei isolated from chick fibroblasts at 0, 10, and 20 min. Chick fibroblasts were grown in 3-2-L roller bottles as described in Materials and Methods. Actinomycin D was added to two roller bottles to a concentration of 0.1 μg/mL, while the untreated bottle was the control. Media was poured off at the times indicated. The cells were washed with ice-cold Tris-buffered saline, cells harvested, nuclei isolated and assayed as described in Materials and Methods. Control RNA polymerase activities were: (I) 104; (II) 231; (III) 58.7 pmol incorporated per 10 min per mg of DNA. RNA polymerase I (■-■); RNA polymerase II (●—●); RNA polymerase III (▲—▲). Inset: RNA polymerase I activity after actinomycin D. Ordinate: percent control activity (log scale) and abscissa: time (min).

TABLE I: Rapid Effect of Actinomycin D and Cycloheximide in Vivo on Rat Liver Nuclear RNA Polymerases Assayed in Vitro.[a]

Treatment[c]	Nuclear RNA polymerase[d] (%)	
	II	I + III
Control	206 ± 5.6 (100)	157 ± 10.2 (100)
Actinomycin D (0.1 μg)	172 ± 16.1 (83)	105 ± 7.7 (67[b])
Control	241 ± 7.59 (100)	241 ± 10.8 (100)
Cycloheximide (100 μg)	281 ± 2.36 (117[b])	160 ± 15.6 (66[b])

[a] Preweaned rat pups were injected with actinomycin D or cycloheximide (different experiments) and, 15 min later, animals were killed, livers removed, nuclei isolated, and RNA polymerases assayed as described in Materials and Methods. Actinomycin D and cycloheximide experiments were performed on separate days. [b] $p < 0.02$. [c] Cycloheximide and actinomycin D were injected intraperitoneally on a μg/g body weight basis and animals were sacrificed 15 min later. [d] Activity is expressed as pmol of UMP incorporated per 5 min per mg of DNA. Each group represents the mean ± SEM of three animals per point.

vivo to see if there was any possible effect on nuclear RNA polymerases as might be expected from a previous study on the effect of actinomycin D on nuclear RNA polymerases in vitro (Lindell, 1976). If actinomycin D affects rRNA synthesis in vivo by selectively inhibiting nucleoplasmic transcription, then low concentrations of this compound injected into animals should exhibit an inhibition of nucleoplasmic transcription with a concomitant but indirect effect on nucleolar transcription. Figure 1 demonstrates that actinomycin D, injected intraperitoneally into rat pups in the indicated low doses per g of body weight, produces a concentration-dependent inhibition of nucleoplasmic RNA polymerase II activity similar to that observed in vitro. RNA polymerase II activity was measured by the assay of liver nuclei isolated 1 h after administration of the drug.

Since actinomycin D acts rapidly in inhibiting rRNA transcription in vivo, the effect of 0.1 μg/g of body weight actinomycin D was examined on the activity of liver nuclear RNA polymerases 15 min after injection of drug. Table I shows that RNA polymerase II is inhibited to 83% of control, while RNA polymerase I activity is 67% of control. Table I also presents the effect of cycloheximide on liver nuclear RNA polymerases 15 min after injection of drug. Nucleoplasmic RNA polymerase II is somewhat higher than control but RNA polymerase I is 66% of control. Examination of additional time points in rat liver nuclei at 30, 45, and 60 min indicates that the $t_{1/2}$ of RNA polymerase I activity is about 30 min (Lindell, 1977).

Solubilization of RNA polymerases from nuclei isolated after in vivo administration of actinomycin D reveals that there is no difference in the amount of RNA polymerase I, II, or III activity when these enzymes are assayed on exogenous template. In addition, no change in the amount of RNA polymerase I is seen after solubilization of the enzyme from liver nuclei after cycloheximide treatment in vivo and assay on exogenous template (data not shown).

Effect of 0.1 μg/mL Actinomycin D on the Time Course of the Inhibition of Nuclear RNA Polymerases in Chick Fibroblasts. Since it is difficult to be assured of the concentration and distribution of actinomycin D in the tissues of animals where inhibition of RNA synthesis is desired, cells in culture were employed to determine how rapidly RNA polymerases are affected after the administration of actinomycin D. Figure 2 presents the effect of a single concentration of actinomycin D (0.1 μg/mL) on the activity of RNA polymerases assayed in nuclei isolated from chick fibroblasts 10 and 20 min later. It can be seen that there is partial inhibition of nucleoplasmic RNA polymerases II while there is a marked effect on the activity of RNA polymerase I. No change was observed in the activity of RNA polymerase III. When RNA polymerases were solubilized from these nuclei and assayed on exogenous DNA template, no change in the amount of RNA polymerase I, II, or III activity was found (data not shown). When the RNA polymerase I activity in nuclei was plotted as log percent activity vs. time, a straight line was obtained with an apparent $t_{1/2}$ of about 20 min (insert Figure 2). This experiment was performed twice and identical results were obtained when expressed as percent of control RNA polymerase activities, although the absolute level of RNA polymerases varied somewhat between the two experiments.

Inhibition of Rapidly Labeled Nuclear Proteins Is Dependent upon the Concentration of Actinomycin D Employed. If actinomycin D has an effect on nucleoplasmic transcription of mRNA and this mRNA has a rapid turnover, there should be a concentration dependent inhibition of rapidly labeled proteins as predicted from the in vitro and in vivo inhibition of nucleoplasmic transcription (Lindell, 1976, and Figure 1).

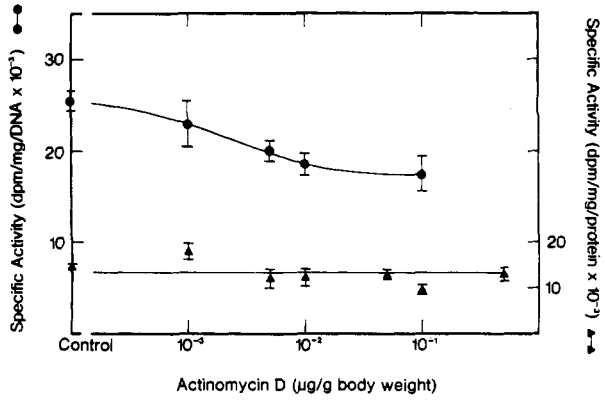

FIGURE 3: Effect of actinomycin D in vivo on the inhibition of rapidly labeled nuclear proteins. Actinomycin D was administered intraperitoneally in concentrations μg/g of body weight and pulsed with 25 μCi of ^{3}H-labeled amino acid mixture 15 min later, and animals were killed 30 min after the initial actinomycin D injection. Livers were extensively washed (10 mL of cold 0.25 M sucrose in buffer A) and nuclei isolated as described in Materials and Methods. Nuclei were resuspended, spotted to Whatmann 3 MM filters, and washed as described in Materials and Methods. Each point represents the average ± SEM of four animals relative to the amount of DNA determined in each nuclear sample. Specific activity of labeled nuclear protein (●—●). Cytoplasmic protein was spotted to Whatmann 3 MM filters and washed with trichloroacetic acid as described in Materials and Methods. Each point represents the average ± SEM of six animals (two experiments). Samples were counted in a Searle Mark III to obtain dpm. Specific activity of cytoplasmic protein (▲—▲). At 0.005 and 0.01 μg/g of actinomycin D, incorporation was significantly different from control (p <0.05).

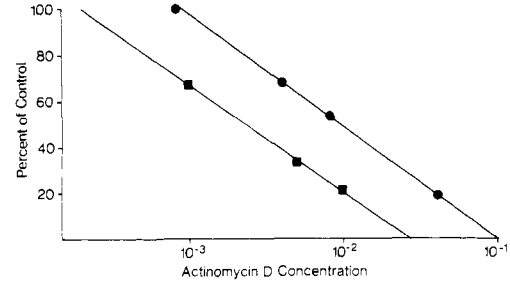

FIGURE 4: Correlation of the inhibition of nuclear protein labeling by actinomycin D in vivo to the inhibition of nucleoplasmic RNA polymerase II in rat liver nuclei in vitro (Lindell, 1976). (●) Nucleoplasmic RNA polymerase II in vitro. (■) Nuclear protein labeling in vivo. Displacement of the nuclear protein labeling curve indicates that the effective concentration is 4.35 times higher when actinomycin D is administered to these animals intraperitoneally.

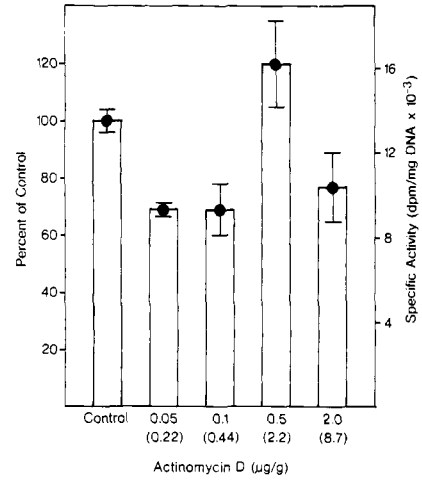

FIGURE 5: Effect of high concentrations of actinomycin D on the synthesis of nuclear proteins. Actinomycin D was injected intraperitoneally into animals as indicated, 10 μCi of ^{14}C-labeled amino acid mixture was injected 15 min later, and nuclei were isolated and counted as described in the legend of Figure 3. Each point represents the average ± SEM of four animals per point. At 0.05 and 0.1 μg/g of actinomycin D, p <0.05. Numbers in parentheses refer to the corrected concentration of actinomycin D multiplied by the factor 4.35 as determined in Figure 4.

Figure 3 presents this study where it can be seen that there is an actinomycin D concentration-dependent inhibition of rapidly labeled nuclear proteins in the range 0.001–0.1 μg/g. Maximum inhibition was observed at an actinomycin D concentration of 0.1 μg/g of body weight. To ascertain whether there is any effect on protein synthesis in general, duplicate samples of cytoplasm remaining from the nuclear isolation procedure were counted. One set (triplicate) was washed with trichloroacetic acid while the other was counted directly. Subtraction of this washed sample from the unwashed companion sample was used as a measure of the amino acid pool size. These data indicate little or no inhibition of total protein synthesis occurred (Figure 3) nor was there any change in amino acid pool size across the concentration range of actinomycin D studied (data not shown). The inhibition of nuclear protein labeling compared with total incorporated label (nuclear and cytoplasmic) represents 5% at 0.1 μg/g of actinomycin D.

When the inhibition of nuclear protein labeling in vivo is compared with the concentration range of actinomycin D observed to inhibit RNA polymerase II in rat liver nuclei in vitro, there was a parallel inhibition but these two observations were not superimposable (Figure 4). The concentration of actinomycin D at which 50% of nucleoplasmic transcription in isolated nuclei is inhibited is 10^{-2} μg/mL while the inhibition of the synthesis of nuclear proteins occurs at 2.3×10^{-3} μg/g of body weight. If these two inhibitions are related, data obtained in the in vivo system (rats) occur at concentrations 4.35-fold lower in concentration than that observed in vitro.

Effect of Higher Actinomycin D Concentrations on the Specific Activity of Nuclear Protein Labeling. When higher doses of actinomycin D (0.5–5 μg/g) are injected into animals and nuclear protein labeling is studied, it can be seen that there is an anomalous "superinduction" of the labeling of these proteins at 0.5 μg/g (Figure 5). When these concentrations are adjusted by the factor 4.35, it can be seen that this anomalous elevated labeling corresponds to concentrations of actinomycin D known to "superinduce" the translation of a number of proteins. The specific activity of nuclear protein labeling was the same at 2 μg/g as at 5 μg/g (data not shown).

Discussion

Studies with actinomycin D have revealed that this compound selectively inhibits rRNA transcription in vivo; however, these same concentrations (0.001–0.01 μg/mL) do not inhibit nucleolar transcription in vitro. It was found that actinomycin D in this concentration range effected only nucleoplasmic RNA transcription by RNA polymerase II in isolated nuclei. From this finding, the suggestion was made that actinomycin D has a selective action in inhibiting unique mRNAs which code for proteins needed for the maintenance of rRNA transcription in vivo (Lindell, 1976).

If the above model for rRNA transcription is correct, three findings should emerge from the administration of low concentrations of actinomycin D in vivo. First, one should observe a slight inhibition of nucleoplasmic RNA transcription when nuclei are assayed in vitro for RNA polymerase II activity; second, there should be more marked (indirect) effect on

rRNA transcription (RNA polymerase I) as assayed in nuclei in vitro; and third, if actinomycin D selectively effects mRNA transcription and this mRNA is rapidly turning over, an effect should be observed on the synthesis of rapidly labeled proteins which migrate to the nucleus.

Figure 1 indicates that when low concentrations of actinomycin D (μg/g of body weight) are administered to small rat pups, the assay of isolated liver nuclear RNA polymerases reveals a low but consistent inhibition of RNA polymerase II. This indicates that actinomycin D administered in vivo is still bound to the DNA of nuclei isolated from the livers of animals treated 1 hr earlier. Elkind et al. (1968) have observed that the half-time of labeled actinomycin D release from cells in culture is about 2.3 h also suggesting that the drug remains firmly bound to nuclear DNA. In addition, Perry & Kelley (1970) observed a linear uptake of labeled actinomycin D in the concentration range (0.001–0.1 μg/mL) of actinomycin D which inhibits rRNA transcription in eukaryotic cells. This finding precludes the possible selective uptake and/or sequestration of actinomycin D in this concentration range by cells in culture.

When liver nuclei were assayed from animals which had been treated with 0.1 μg/g of body weight actinomycin D, it can be seen that RNA polymerases I plus III activities are effected more than RNA polymerase II (Table I). While this effect could be due to a direct inhibition of rRNA transcription by actinomycin D, from the previous in vitro results, it is suggested that this concentration primarily effects nucleoplasmic transcription (Lindell, 1976). It is of interest that cycloheximide produced the same effect on RNA polymerase I activity within the same time period. It is proposed that both of these drugs inhibit rRNA transcription indirectly. The stimulation of RNA polymerase II activity by cycloheximide was not expected; however, others have observed increased mRNA accumulation after cycloheximide administration to cells in culture (Johnson & Meister, 1977; Maroun & Miller, 1977).

To gain a more quantitative assessment of the effect of actinomycin D on the time course of nuclear RNA polymerases, actinomycin D was added to cultures of chick fibroblasts. Nuclei isolated from treated cells and assayed for RNA polymerase activities reveal that there is an effect on both RNA polymerase I and II (Figure 2). It would appear that RNA polymerase II is inhibited to a constant level while the effect on RNA polymerase I is time dependent. The $t_{1/2}$ of RNA polymerase I activity (20 min), coupled with the finding that the absolute level of these enzymes is unchanged in assays of solubilized nuclear protein on exogenous template, suggests the possibility that RNA polymerase I is unable to reinitiate transcription at low actinomycin D concentrations.

Additional evidence relating the concentration dependent effect of actinomycin D on nuclear protein labeling (Figure 3) suggests that there is a close temporal relationship to the synthesis of these nuclear proteins (but not cytoplasmic proteins) and the activity of nucleolar RNA polymerase I. Since there is a well-known relationship between continued protein synthesis and rRNA transcription (Holland, 1963; Fiala & Davis, 1965; deKloet, 1966; Higashi et al., 1968; Willems et al., 1969; Muramatsu et al., 1970; Craig & Perry, 1970; Cooper & Gibson, 1971) and the activity of RNA polymerase I in isolated nuclei (or nucleoli) (Muramatsu et al., 1970; Yu & Feigelson, 1971; Higashinakagawa & Muramatsu, 1972; Lampert & Feigelson, 1974; Gross & Pogo, 1974; Cereghini & Franze-Fernandez, 1974; Chesterton et al., 1975; Gross & Pogo, 1976), it is suggested that actinomycin D is inhibiting the mRNA needed for the proteins required for rRNA transcription. For actinomycin D to have such a rapid effect on both the labeling of these proteins and rRNA transcription, the mRNAs for these proteins are predicted to have an extremely rapid rate of turnover.

Since higher concentrations of actinomycin D are known to cause superinduction of a large number of proteins (Tomkins et al., 1972), it is possible that the anomalous effect observed at a dose of 0.5 μg/g of actinomycin D (Figure 5) is related to superinduction especially since in vivo doses appear to be present in higher concentrations (Figure 4). If rapidly turning over mRNAs are required for the continued transcription of rRNA, it is possible that the superinduction observed here involves the stabilization of these mRNAs from turnover. However, Killewich et al. (1975) were unable to demonstrate that the mRNA for rat liver tryptophan 2,3-dioxygenase is elevated after an actinomycin D injection (4 μg/g of body weight) known to cause superinduction of this enzyme. Others have suggested that superinduction involves the stabilization of proteins from turnover (Reel & Kenney, 1968; Kenney et al., 1973). In this case, it is not likely that these proteins inhibited by actinomycin D are rapidly turning over but rather rapidly utilized.

Maximum inhibition of nuclear protein labeling by actinomycin D occurred at a dose of 0.1 μg/g. This amounts to 30% of the total nuclear protein labeled within this time period. When the concentration-dependent inhibition of nuclear protein labeling is compared with the inhibition of nucleoplasmic transcription in vivo (Figure 3 and Lindell, 1976), there is a displacement to the left which probably indicates that a localized injection of actinomycin D into the peritoneal cavity does not distribute itself throughout the body within this time period (30 min). It can be seen that the difference between these two parallel lines amounts to a 4.35-fold difference in concentration. Therefore, to relate these in vivo studies in rats to the in vitro studies (Lindell, 1976), the concentration injected must be multiplied by this factor if these two observations are related.

The nature of the rapidly labeled proteins inhibited by low concentrations of actinomycin D has yet to be studied in this laboratory. Suskind (1965) has, however, observed a rapid effect (within 15 min) of actinomycin D (0.05 μg/mL) on the labeling of nucleolar, but not cytoplasmic or nucleoplasmic, proteins in HeLa cells by autoradiography. Further, Suskind found that 28% of nuclear protein labeling was found in the nucleolus. This value is close to that observed in this study (Figures 3 and 5) where a maximum 30% inhibition of nuclear protein labeling was observed. In addition, Hnilica et al. (1966) have shown that nucleolar proteins are among the most rapidly labeled nuclear proteins after a pulse with labeled amino acids.

Lastick & McConkey (1976) have recently demonstrated that a low concentration of actinomycin D (0.04 μg/mL) inhibited the "exchange" of a group of rapidly labeled (30 min) basic polypeptides in the 40S ribosomal subunit of HeLa cells. These authors suggest that these proteins are not "exchanged" with a pool of preexisting ribosomal precursor proteins in the presence of actinomycin D, but it is equally plausible that the action of actinomycin D involves inhibition of the transcription of mRNAs which code for the translation of these peptides.

Others have described the effect of low doses of actinomycin D on the suppression of nucleolar protein labeling (Maisel & McConkey, 1971) or ribosomal subunit peptides (Tsurugi et al., 1972; Tsurugi & Ogata, 1977). Tsurugi & Ogata (1976) and Warner & Gorenstein (1977) have described the in vitro translation of ribosomal subunits from mRNA. Wu et al. (1977) have shown that there is a close relationship between

the transcription of polyadenylated mRNA for ribosomal subunits and rRNA transcription in normal liver, regenerating liver, and Novikoff hepatoma. Roth et al. (1976) have observed that HeLa ribosomal proteins are rapidly taken up by isolated nuclei where they are localized in nucleoli.

These results, and others cited above, suggest a close temporal relationship to the coupled transcription of mRNA and the translation of rapidly labeled proteins which migrate to the nucleolus. It would appear that these proteins are, in fact, ribosomal subunit peptides whose continued translation is required for the maintenance of rRNA transcription. In this regard, Gorenstein & Warner (1976) have observed the coordinate synthesis of mRNA and ribosomal subunits in yeast. These authors describe a temperature sensitive mutant yeast strain which is not capable of synthesizing a large number of ribosomal subunit proteins at the nonpermissive temperature and that this is influenced directly by the lack of mRNA transcription. The half-life of the inhibition of the synthesis of ribosomal subunits is near that observed in this study using actinomycin D and measuring nucleolar RNA polymerase I activity.

These results provide further evidence for an extranucleolar control of rRNA transcription in eukaryotes and suggest that actinomycin D is an effective inhibitor of rRNA transcription via this mechanism. Although we do not demonstrate a direct inhibitory effect on mRNA synthesis, a number of others have shown that nucleoplasmic or presumptive mRNA is inhibited by these low concentrations of actinomycin D (Penman et al., 1968; Perry & Kelley, 1970; Lindberg & Persson, 1972).

Acknowledgments

The authors would like to thank the following individuals who assisted in these experiments: Sandra Burt, Royal Ellinger, David Sundheimer, Dorothy Warren, James Warren, and Dr. Marshall Dinowitz who supplied the cells. The authors would also like to thank Dr. Norman Brink of Merck and Co. who arranged for the gift of actinomycin D.

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Hormonal Regulation of Hemoglobin Synthesis in Cells of Fetal Calf Liver Cultured in a Serum-Free Medium†

L. F. Congote* and Samuel Solomon

ABSTRACT: A system of short-term cultures of fetal calf liver in a serum-free medium is described in which the effects of hormones and drugs on hemoglobin synthesis can be tested directly without interference from fetal calf serum normally used for this type of cell culture. In the model system described, hemoglobin synthesis was followed using the incorporation of ^{59}Fe into hemoglobin-associated heme or the incorporation of [^{3}H]leucine into fetal hemoglobins after their fractionation by acrylamide gel electrophoresis. Erythropoietin (0.2 μg/mL) stimulated ^{59}Fe incorporation into heme associated with hemoglobin in cell cultures isolated from fetuses of all the gestational ages studied, namely from 90 to 175 days. Testosterone (10^{-8} M) was active only in cell cultures from fetuses of 115 to 155 days of gestation, while isoproterenol (10^{-10} M) was active only in liver cells from fetuses of 115 to 135 days of gestation. Etiocholanolone (10^{-8} M) had an effect similar to that of testosterone on hemoglobin synthesis but this was not significantly different from control values. At 115-180 days of gestation, the synthesis of adult hemoglobin in fetal liver cells is almost undetectable. There are two main fetal hemoglobins synthesized in vitro which have not been previously described. The first one (F_0) represents 50 to 70% of the fetal hemoglobin in vitro after a 5-h incubation with [^{3}H]leucine and is the main fetal hemoglobin of circulating red cells. F_0 is also the major hemoglobin synthesized by liver cell cultures after 16 to 24 h of incubation with [^{3}H]leucine. The fetal hemoglobin F_1 is only occasionally found in circulating red cells and when present it is less than 15% of the total hemoglobin. F_1 is found in the fetal liver in amounts of 15 to 50% of the total and is synthesized in large amounts in cell cultures in vitro using short incubation times with [^{3}H]leucine (5 h or less). Both testosterone and erythropoietin stimulated preferentially the synthesis of F_0. These results demonstrate that both hormones are not only stimulating total hemoglobin synthesis but also accelerating specifically the synthesis of the main fetal hemoglobin of mature red cells (F_0).

The human fetal liver is the main site of hemoglobin synthesis at midterm. The main hemoglobin being synthesized at this period of gestation is fetal hemoglobin ($\alpha_2\gamma_2$), although adult hemoglobin synthesis already represents 10% of the total hemoglobin synthesized (Kan et al., 1972; Hollenberg et al., 1972; Lanyon et al., 1975). It has been found that several hormones and drugs such as erythropoietin, testosterone, and isoproterenol stimulate the synthesis of hemoglobin or heme associated with hemoglobin in cell cultures of human fetal liver and that these hormones or drugs are active only at certain discrete periods of fetal development (Basch, 1972; Congote et al., 1974; Shchory and Weatherall, 1975; Congote and Solomon, 1977; Congote, 1977). Because the availability of human fetuses is very limited, further investigations in this field required the development of an experimental model with a pattern of fetal erythropoiesis similar to man. We chose the calf fetus as a model because it not only synthesizes fetal hemoglobin at midterm but also has the switch from fetal to adult hemoglobins similar to humans (Huisman, 1974; Kitchen and Brett, 1974). In this paper we describe the effects of a number of hormones on hemoglobin synthesis in fetal calf liver cells. We were able to test these hormones in liver cells cultured in a serum-free medium, thus excluding any interaction with fetal calf serum which was a drawback of the media previously employed in such studies.

Materials and Methods

Materials. All materials for tissue culture were purchased from Flow Laboratories. Bovine serum albumin (fraction V) and DL-isoproterenol were purchased from Sigma, and bovine transferrin was from Miles or Calbiochem. Sheep plasma erythropoietin (step III) was purchased from Connaught Laboratories, Toronto, Canada. The erythropoietin preparations used had specific activities of 2.2, 4.5, and 5 units/mg. $^{59}FeCl_3$ (specific activities 10-25 mCi/mg of Fe) was obtained from Frosst-Merck Laboratories, Montreal, Canada. L-[4,5-^{3}H]Leucine (53-60 Ci/mmol) and L-[U-^{14}C]leucine (330

† From the Departments of Biochemistry, Experimental Medicine, and Obstetrics and Gynecology, McGill University and the McGill University Clinic, Royal Victoria Hospital, Montreal, Canada. *Received August 17, 1977.* Supported by a grant from the Conseil de la Recherche en Santé du Québec and also in part by Grants MA-6072 and MT-1658 from the Medical Research Council and Grant HDO-4365 from the National Institutes of Health. L.F.C. is a Scholar of the Medical Research Council of Canada.

0006-2960/78/0417-1160$01.00/0