Growth Hormone and Drug Metabolism

ACUTE EFFECTS ON NUCLEAR RIBONUCLEIC ACID POLYMERASE ACTIVITY AND CHROMATIN

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1. Adult male rats, subjected either to sham operation or to hypophysectomy and adrenalectomy were maintained for 10 days before treatment with growth hormone. Results of the acute effects of growth hormone on the rat liver nuclear RNA polymerase I (nucleolar) and II (nucleoplasmic) activities as well as the chromatin template capacity were then studied and compared with the growth-hormone effects on the drug metabolism described in the preceding paper (Wilson & Spelsberg, 1976). 2. Conditions for isolation and storage of nuclei for maintenance of optimal polymerase activities are described. It is verified that the assays for polymerase activities require a DNA template, all four nucleoside triphosphates, and a bivalent cation, and that the acid-insoluble radioactive product represents RNA. Proof is presented that under high-salt conditions DNA-like RNA (polymerase II) is synthesized, and that under low-salt conditions in the presence of α -amanitin, rRNA (polymerase I) is synthesized. 3. In the livers of hypophysectomized/adrenalectomized rats, growth hormone increases the activity of both RNA polymerase enzymes and the chromatin template capacity within 1 h after treatment. The effects last for 12 h in the case of polymerase II but for only 6 h in the case of polymerase I. Sham-operated rats respond to growth hormone in a manner somewhat similar to that shown by hypophysectomized/adrenalectomized rats. These results, which demonstrate an enhancement of RNA polymerase I activity in response to growth hormone, support those from other laboratories. 4. Growth-hormone enhancement of the chromatin template capacity in the liver of hypophysectomized/adrenalectomized rats contrasts with previous reports. The growth-hormone-induced de-repression of the chromatin DNA could represent the basis of the growth-hormone-induced enhancement of RNA polymerase II activity in the hypophysectomized/adrenalectomized rats, although some effect of growth-hormone on the polymerase enzymes is still suggested. 5. No correlation is observed between the transcriptional responses to growth hormone and activities of the mixed-function oxidases. Such a correlation is not necessarily expected, since the latter enzymes represent only a fraction of the total proteins being synthesized in the cell. The growth hormone-induced RNA polymerase activity and derepression of the DNA probably represent the synthesis of protein involved in liver growth. 6. It is concluded that the growth-hormone-induced growth in the liver of hypophysectomized/adrenalectomized rats is preceded by increases in RNA polymerase II activities and a de-repression of the chromatin DNA. Since these effects are detected within minutes after growth-hormone treatment and before growth-hormone-induced increases in general protein synthesis and some enzyme activities are noted, it is proposed that growth-hormone action not only requires transcriptional response but this response may represent a primary site of action in the cell. The cellular entities or mechanisms which growth hormone uses to alter transcription in the nucleus remain to be determined.

It is quite well established that growth hormone markedly affects the protein-synthesizing activity of its target cells (Korner, 1965). When the hormone is administered *in vivo*, an enhancement of protein

synthesis is observed *in vivo* or *in vitro* with isolated ribosomal fractions. However, when growth hormone is added *in vitro* to isolated ribosomal fractions, there is no effect on protein synthesis. These results support the idea that growth hormone initially acts in areas of the cell other than those where protein synthesis takes place. The growth hormone may alter the activities of

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many enzymes by altering synthesis and possibly turnover of the enzymes themselves (as has been shown for ornithine decarboxylase) (Jänne *et al.*, 1968; Jänne & Raina, 1969; Russell *et al.*, 1970). One of the earliest metabolic events observed after the administration of growth hormone to animals is the stimulation of cellular RNA synthesis (Korner, 1964; Talwar *et al.*, 1964; Salaman *et al.*, 1972; Jackson & Sells, 1968; Drews & Brawerman, 1967; Ip & Brossard, 1971). By using inhibitors of protein and RNA synthesis, Russell *et al.* (1970) demonstrated that growth-hormone-induced increases in ornithine decarboxylase activity in rat liver are dependent primarily on RNA synthesis. Further, this action of growth hormone on gene transcription occurred within 2h after injection of growth hormone into adult or weanling rats. These results, together with the fact that growth-hormone-induced synthesis of total cell protein can be obliterated with appropriate actinomycin D treatment (Tata, 1970), support the idea that growth hormone manifests its action on various enzymes through the alteration of gene transcription. Further studies have shown that growth-hormone stimulation of RNA involves all species of RNA but most significantly the ribosomal RNA (Talwar *et al.*, 1964; Salaman *et al.*, 1972; Drews & Brawerman, 1967; Ip & Brossard, 1971; Tata, 1970; Breuer & Florini, 1966). Other studies of the mechanism of this stimulation of RNA synthesis have indicated an effect of growth-hormone treatment on the nucleolar polymerase I (which synthesizes ribosomal RNA) with no observable effect on the extranucleolar polymerase II (synthesizing DNA-like RNA) (Jänne & Raina, 1969; Smuckler & Tata, 1971) or on the DNA restriction in the chromatin (Breuer & Florini, 1966; Gupta & Talwar, 1968). The present study was designed to examine further the transcriptional responses to early growth-hormone action. It is the long-range goal to determine whether or not early metabolic events which are associated with growth-hormone action in rat liver may be explained by early effects on nuclear transcription.

Methods and Materials

Animals and treatment

Male Sprague-Dawley rats were obtained from a local supplier (Zivic-Miller Inc.) and treated exactly as described in the preceding paper (Wilson & Spelsberg, 1976). Animals were injected once subcutaneously with 5 mg of pig growth hormone (Sigma Chemical Co., St. Louis, Mo., U.S.A.; 0.8–1 i.u./mg) dissolved in 0.1 M-NaHCO₃. This concentration of growth hormone was found essential for maximal acute effects on drug metabolism as well as for prolonged (3-day) effects. Control rats received only the vehicle (NaHCO₃). No difference was observed when 5.0 mg of bovine albumin as opposed to the

NaHCO₃ alone was injected into control animals (Wilson, 1973). In addition, all experiments were performed during the same hours of a day and on animals which were on consistent diet and light/dark cycle.

Isolation of tissue and nuclei

Pooled livers were minced on ice, weighed and used for the isolation of nuclei to be analysed for RNA polymerase activities as described previously (Spelsberg *et al.*, 1974; Glasser *et al.*, 1972).

Assay of endogenous DNA-dependent RNA polymerase activity

Assay conditions used in these experiments were derived from a series of experiments aimed at achieving optimal enzyme activity per nucleus (based on the incorporation of labelled UMP into an acid-insoluble product per mg of DNA) (Spelsberg *et al.*, 1974; Glasser *et al.*, 1972; Glasser & Spelsberg, 1972). These studies are described in the Results section. The radioactivity (c.p.m.)/mg of DNA was calculated from the radioactivity (c.p.m.)/filter and the μ g of DNA added to the reaction.

Isolation of chromatin

Nuclei to be used for the isolation of chromatin were further purified by dilution of the nuclei solution with 10 vol. of a solution containing 0.5 M-sucrose in TKM buffer (50 mM-Tris/HCl/25 mM-KCl/2 mM-MgCl₂, pH 7.5) containing 0.2% (v/v) Triton X-100 (Spelsberg & Hnilica, 1971; Spelsberg *et al.*, 1971). The homogenate was filtered through organza cloth (100 mesh) and centrifuged for 10 min at 10000g in a Sorvall RC-2B centrifuge. These nuclear pellets were then subjected to a series of hypotonic buffer extractions to obtain chromatin as described elsewhere (Spelsberg & Hnilica, 1971; Spelsberg *et al.*, 1971).

Analysis of the composition and template capacity of the rat liver chromatins

The analysis of DNA and template capacity of isolated chromatin has been described in detail previously (Spelsberg *et al.*, 1971; Spelsberg & Hnilica, 1971). In the determination of the capacity of each of the chromatin preparations to serve as a template for DNA-dependent RNA synthesis *in vitro*, the amounts of bacterial enzyme and DNA (chromatin) per reaction were selected to establish assay conditions in which the template is rate-limiting and the incorporation of [¹⁴C]UMP into RNA was linear over the 10 min assay period (see the Results section).

Base composition of the RNA product

An estimation of base composition of the RNA synthesized by the nuclei under low-salt and high-salt

conditions was achieved by using the approach of Pogo *et al.* (1967). In the present experiments the standard reaction assay mixture described above was used except either [^{14}C]UTP (15 $\mu\text{Ci}/\mu\text{mol}$) or [^{14}C]GTP (22 $\mu\text{Ci}/\mu\text{mol}$) was substituted for [^3H]UTP. The acid-insoluble products on the filters were counted for radioactivity as described above and the amounts of GMP and UMP (in nmol) incorporated into RNA under the various conditions calculated. The ratios of UMP to GMP were then calculated to characterize the RNA, as described previously by Pogo *et al.* (1967).

Results

Isolation of rat liver nuclei

The isolation of liver nuclei with maximal endogenous RNA polymerase activity was investigated. More commonly used methods of nuclei isolation involving solutions of low sucrose concentration (0.25M) and non-ionic detergents (Triton X-100) resulted in nuclei with lower RNA polymerase activity (especially in the nucleoplasmic polymerase II) than found with the procedure described in the Methods and Materials section. The diffusion of polymerases or some factors from the nuclei during exposure to lower sucrose concentrations is suggested as an explanation for the lower values. The nuclei isolated by the procedure described in the Methods and Materials section does show, by phase and electron microscopy, contamination with minimal cytoplasmic debris and with the outer membrane of the nuclear envelope with attached ribosomes. Further purification of the nuclei by discontinuous-sucrose-density-gradient centrifugation resulted in low nuclear yields and decreased polymerase activity per mg of DNA (nuclei). Consequently the procedure described in the Methods and Materials section was selected on the basis of maximum enzyme yield per nucleus and for procedural simplicity, since large numbers of nuclei preparations (as high as 90/24h period) were required. These results with liver nuclei, closely simulate those obtained with rat uterine nuclei (Glasser *et al.*, 1972) and chick oviduct nuclei (Spelsberg *et al.*, 1974; Knowler *et al.*, 1973).

Properties of the DNA-dependent RNA synthesis in isolated rat liver

Investigations were performed to determine whether or not our assays were truly measuring the DNA-dependent RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6). Actinomycin D and DNAase (deoxyribonuclease) pretreatment of nuclei greatly decreased the incorporation of nucleotides, thus showing the requirements for a DNA template. Since the radioactive product was found to be labile to RNAase (ribonuclease) but not to DNAase or Pronase, and since

the majority of the labile product was made completely acid-soluble by hydrolysis in 0.3M-KOH for 1h at 37°C, the radioactive product of the assay probably represents RNA. The RNAase activity endogenous to all nuclei was found to be one of the primary causes of the lack of linearity of nucleotide incorporation (RNA synthesis) by isolated nuclei. The RNAase activity is present in more purified nuclei preparations devoid of all cytoplasmic debris and in nuclei without the outer membrane. None of the commonly used RNAase inhibitors, e.g. polyvinyl sulphate, bentonite etc., were able to completely eliminate the nuclear activity. However, the use of a reaction temperature of 15°C, as suggested by Chambon *et al.* (1968, 1970) and verified by Roeder & Rutter (1970) did eliminate all detectable RNAase activity (see Table 1) and resulted in a linear incorporation of nucleotides for 20min or more (see Table 2). Consequently, all assays were performed for 10min at 15°C. Because there were large numbers of nuclei preparations to be analysed, storage of the nuclei in glycerol solution at -20°C for several

Table 1. Analysis of RNAase activity in liver nuclei of rats under low- and high-salt conditions

Equal amounts of [^3H]RNA (12000 c.p.m./ μg) were incubated with 100 μg of DNA (as nuclei) at 37° or 15°C in the standard polymerase reaction mixture without the nucleotides. Commercial RNAase was also used in some reactions to test the methodology. After 30min, 200 μg of unlabelled yeast RNA was added, followed by 1ml of a cold solution containing 10% (w/v) trichloroacetic acid/0.1% sodium pyrophosphate; the solution was left at 4°C for 20min, then centrifuged for 10min at 10000g. The pellets were rinsed in cold 5% (v/v) trichloroacetic acid and then collected on Millipore filters (0.45 μm pore size) with cold trichloroacetic acid solution. The filters were dried, washed in toluene, placed in a standard scintillation mixture containing PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene], and then counted for radioactivity in a liquid-scintillation spectrometer. Portions (200 μl) of the supernatant of the original trichloroacetic acid precipitation were spot dried on Millipore filters and the filters treated as described above.

	Radioactivity (c.p.m./ml of incubation media)	
	Acid- soluble	Acid- insoluble
Low salt (nuclei+[^3H]RNA)		
Incubated at 15°C	25	1640
Incubated at 37°C	1210	550
High salt (nuclei+[^3H]RNA)		
Incubated at 15°C	15	1750
Incubated at 37°C	830	810

Table 2. *Endogenous RNA polymerase activity in isolated liver nuclei of normal rats assayed at 15° and 37°C for various time-intervals*

Stored nuclei were kept in 25% (v/v) glycerol/buffer medium (described in the Methods and Materials section) for 2 months at -20°C and then re-assayed. All components, including α -amanitin (0.2 μ g/reaction mixture) were added together (see the Methods and Materials section) and the reactions started by the addition of the nuclei. Radioactivity is expressed as the mean of three replicate determinations. The temperatures listed represent those used in the assays for polymerase activity.

Temperature ...	Radioactivity [c.p.m./mg of DNA (as nuclei)]			Inhibition by α -amanitin (%) (stored nuclei) (15°C)
	Fresh nuclei		Stored nuclei (15°C)	
	15°C	37°C		
Low salt				
Without α -amanitin	—	—	1571	
10min	—	—		
With α -amanitin				
5min	491	816	488	
10min	1098	1160	1100	30
20min	1680	1087	1710	
High salt				
Without α -amanitin				
5min	5030	12000	4920	
10min	10050	16400	10140	
20min	19400	18500	19250	
With α -amanitin				
10min			1521	85

months was necessary; however, no significant change on the activity of endogenous polymerase I or II (see Table 2 for an example) was observed. The stability of polymerase activity of nuclei in glycerol accords with previous reports (Spelsberg *et al.*, 1974; Glasser & Spelsberg, 1972; Read & Mauritzen, 1970). In the following experiments involving growth hormone, all nuclei preparations were stored from 1 to 6 days before being assayed for polymerase activity.

To test whether our measurements in low-salt conditions with α -amanitin were indicative of the nucleolar polymerase activity (or polymerase I, synthesizing ribosomal RNA) and whether the results obtained in high-salt conditions were a measurement of nucleoplasmic polymerase activity (or polymerase II, synthesizing DNA-like RNA), two approaches were taken. First, the effect of α -amanitin, an inhibitor specific for the nucleoplasmic polymerase II (Stirpe & Fiume, 1967; Novello & Stirpe, 1969; Keding *et al.*, 1970; Jacob *et al.*, 1970), on the enzyme activities was studied under both low- and high-salt conditions. The concentration of α -amanitin used was selected from titration experiments in which, with increasing concentrations of α -amanitin, the degree of inhibition reached a plateau. Under high-salt conditions, the polymerase activity was decreased by α -amanitin to 15% of the control value (see Table 2). This demonstrates that 85% of the polymerase activity of nuclei under high-salt con-

ditions represents the polymerase II that transcribed DNA-like RNA. Very high concentrations of α -amanitin resulted in a complete inhibition, which probably represents the inhibition of the polymerase III activity. However, under low-salt conditions the enzyme activity is only decreased to 70% of the control value, indicating that 30% of the total enzyme activity in this reaction represents polymerase II activity. Since the polymerase II enzyme contributes as much as 30% of the total enzyme activity of the nuclei under low-salt conditions, α -amanitin (0.2 μ g/reaction) was included in all assays performed under the low-salt conditions to assure measurement of the nucleolar polymerase I activity. The second approach involved the analysis of the base composition as described by Pogo *et al.* (1967). As shown in Fig. 1, by using labelled GTP and UTP, the assays under low-salt conditions with α -amanitin produced RNA which had a UMP/GMP ratio of 0.70–0.74 whereas the high-salt conditions produced RNA with an UMP/GMP ratio of >1.0. Thus, in the low-salt conditions with α -amanitin, primarily ribosomal RNA is synthesized (representing polymerase I activity) whereas in high-salt conditions, DNA-like RNA is synthesized (representing polymerase II activity; Pogo *et al.*, 1967).

The use of Mg^{2+} and Mn^{2+} ions in the same reaction at concentrations suggested by Roeder & Rutter (1970) resulted in the synthesis of RNA with a base composition similar to that found when the ions

Fig. 1. Base-composition analysis of the RNA synthesized by isolated rat liver nuclei under high- or low-salt conditions with α -amanitin

Assays were performed either with $[^3\text{H}]\text{UTP}$ or with $[^3\text{H}]\text{GTP}$ under the two salt conditions. The amount of each nucleotide incorporated (nmol/mg of DNA) was determined and the ratios of UMP to GMP calculated. The top hatched area represents the U/G ratio reported for DNA-like RNA, the bottom hatched area represents that for ribosomal RNA. See the Methods and Materials section for further details.

of only one of the metals were used. Consequently, the ions of both metals were used in subsequent assays to allow the use of one stock reaction mixture. It was also found that higher salt concentrations in the assays, e.g., $0.35\text{M}-(\text{NH}_4)_2\text{SO}_4$, produced RNA with a similar base composition to that of RNA synthesized with $0.25\text{M}-(\text{NH}_4)_2\text{SO}_4$, but the former conditions caused greater nuclear rupture and protein removal from the chromatin DNA. Therefore $0.25\text{M}-(\text{NH}_4)_2\text{SO}_4$ was used for the high-salt conditions.

Diurnal variations and surgical effects on RNA polymerase activity

Fig. 2 shows that the activities of both RNA polymerase I and II from rats which underwent hypophysectomy and adrenalectomy 10 days previously are lower than that from rats which underwent sham operation 10 days previously. This decreased activity is observed throughout the

diurnal period (Fig. 2). The activities of the two polymerase enzymes in both hypophysectomized/adrenalectomized and sham-operated animals display a diurnal variation. Similar results have been reported in the liver of normal (non-operated) rats (Glasser & Spelsberg, 1972). Fig. 2 also shows that the time changes (8:00 a.m.–6:00 p.m.) in polymerase I are discordant in sham-operated as against hypophysectomized/adrenalectomized rats, whereas concordant changes for polymerase II are noted during the daytime. The diurnal variations in the hypophysectomized/adrenalectomized rats could be the result of the habitual activities of the rats. Since there is a diurnal fluctuation in basal activities of both types of polymerases in the livers of sham-operated and hypophysectomized/adrenalectomized rats, all experiments assessing the effect of growth hormone are compared with similarly treated rats injected with the vehicle only. These 'control' rats at each period of observation are used as a reference (100%) for basal enzyme activity.

Fig. 2. Diurnal variations and effects of hypophysectomy and adrenalectomy on the endogenous RNA polymerase activities measured in isolated liver nuclei

The mean and range of three replicate determinations are given. See the Methods and Materials section for a description of the endogenous polymerase assays. ●, Sham-operated rats; ○, hypophysectomized/adrenalectomized rats. (a) Polymerase I activity (rRNA or nucleolar RNA synthesis) (low salt); (b) polymerase II activity (DNA-like RNA synthesis) (high salt).

Table 3. *Effects of growth hormone and the endogenous RNA polymerase II activity (assayed under high-salt conditions) on untreated, sham-operated and hypophysectomized/adrenalectomized rats*

The values in each group are expressed as a percentage of control values given by similarly treated rats injected with vehicle only. The mean and range of three replicate determinations are given. See the Methods and Materials section for details of the treatment of rats and the injection of growth hormone.

Time after growth-hormone injection (h)	RNA polymerase II activity (% of control)		Hypophysectomized/adrenalectomized
	Intact	Sham-operated	
1	126 ± 7	87 ± 3	197 ± 6
2	121 ± 4	110 ± 3	115 ± 5
24	77 ± 5	84 ± 10	71 ± 9
48	80 ± 4	90 ± 5	99 ± 4
72	105 ± 5	103 ± 6	97 ± 5

Growth-hormone effects on RNA polymerase activities

Initial experiments involved the analysis of the effects of the operative procedures alone on the RNA polymerase II activity in growth-hormone-treated rats. In Table 3, for example, it was found that the operation procedures alone (sham-operated rats) caused a different response of the enzyme activity at early periods after injection of growth hormone compared with that of the non-operated animals. At later periods after growth-hormone administration, differences between sham-operated and non-operated rats were not found. As a result of early differences in the growth-hormone effect in non-operated as against sham-operated animals, the responses to growth hormone by the hypophysectomized/adrenalectomized rats were compared with sham-operated rats as opposed to non-operated rats. Table 3 also shows an immediate (1 h) response of the polymerase II activity to growth hormone in the hypophysectomized/adrenalectomized rats, with activity returning to control values only at a much later time (by 48 h). Therefore subsequent experiments involved 1–96 h observations after growth-hormone injection to ensure measurements of all immediate growth-hormone effects on the enzyme activity.

Fig. 3 illustrates the results of a typical experiment involving the effects of growth-hormone administration on the nucleolar RNA polymerase I activity. Two groups of rats, sham-operated and hypophysectomized/adrenalectomized, were injected with growth hormone. Two similarly treated groups of rats were injected with vehicle only and used as controls at each time-period. Growth hormone caused a multiphasic response of the nucleolar polymerase I activity in sham-operated rats. The activity decreased below the control value by 1 h, increased above the control value by 2 h and this was followed by another decrease at 6 h. In the hypophysectomized/adrenalectomized rats, the enzyme activity displayed a similar multiphasic response, with a marked increase over the control value by 1 h

Fig. 3. *Effect of growth hormone on the endogenous RNA polymerase I activities in sham-operated or hypophysectomized/adrenalectomized rats measured in isolated nuclei under low-salt conditions with α -amanitin (nucleolar or ribosomal RNA synthesis)*

Male Fischer rats were injected with growth hormone (5mg/rat) at zero time. Values are expressed as a percentage of the control value obtained with similarly treated rats injected with vehicle only (the animals were killed at the same time-intervals). The mean and range of three replicate determinations are given. ●, Sham-operated rats; ○, hypophysectomized/adrenalectomized rats.

followed by a gradual decrease below the control value by 6 h and another increase in activity (over the control value) by 10 h. In both groups of rats, enzyme activity approached that of control rats (those injected with vehicle only) by 24 h and remained at the control value up to 96 h after growth-hormone injection. The hypophysectomized/adrenalectomized rats compared with the sham-operated animals thus differed in polymerase I activity mainly in the early (1 h) response to growth hormone. In general, polymerase I activity in the hypophysectomized/adrenalectomized rats displayed a greater response to

Fig. 4. Effect of growth hormone on the endogenous RNA polymerase II activity measured in isolated liver nuclei under high-salt conditions (DNA-like RNA synthesis)

For details, see the legend to Fig. 3.

growth-hormone treatment compared with that of the sham-operated rats.

A similar experiment was performed to measure the response of the nucleoplasmic RNA polymerase II activity to growth hormone (Fig. 4). In sham-operated rats, the nucleoplasmic polymerase II activity shows an initial (1 h) decrease below control values whereas at subsequent periods, the activity rises above control values and remains above (although fluctuating) up to 10 h. From 24 to 72 h, the activity drops slightly below control values and rises to control values by 96 h. The polymerase II activity in the hypophysectomized/adrenalectomized rats, however, shows an immediate rise above control values 1 h after growth hormone treatment. This activity remains above control values for 10 h and is greater than that observed in the sham-operated animals. From 24 to 48 h the activity decreases below that of controls, after which the activity returns to control values. In both sham-operated and hypophysectomized/adrenalectomized rats, the nucleoplasmic polymerase II shows a more sustained increase in activity over controls in response to growth hormone than that found for the nucleolar polymerase I.

Growth-hormone effects on chromatin template capacity

The ability of a cell nucleus to synthesize RNA can be limited by various mechanisms such as (1) substrate (nucleotide) concentration, (2) amount of available DNA to serve as a template, (3) amount of active enzyme, and (4) the efficiency of the available enzyme in polymerizing nucleotides. By using an excess of nucleotides in our RNA polymerase assay *in vitro* the first possibility should be eliminated. To assess the growth-hormone effects on the second possibility, experiments were performed to investigate the ability of the liver chromatin from the rats

treated with growth hormone to serve as a template for the DNA-dependent RNA synthesis.

One of the most commonly used and at present most sensitive methods for determining the amount of DNA in chromatin (which can serve as a template for RNA synthesis) is the use of a reaction *in vitro* fortified with exogenous RNA polymerase for DNA-dependent RNA synthesis. A bacterial RNA polymerase which contains all 'factors' (Burgess, 1969) such as the one used in our experiments has been shown to transcribe just about any available DNA, native or synthetic. When the amount of transcribable DNA in chromatin is to be determined, emphasis is placed on the rate-limiting nature of the template in the reaction, and measurements are made during the linear phase of the nucleotide incorporation. Fig. 5(a) shows the incorporation of nucleotides for a 10 min reaction plotted against the amount of DNA template (as chromatin). By selecting a rate-limiting amount of chromatin DNA for these assay conditions, a linear rate of nucleotide incorporation is observed (Fig. 5b). Under these conditions, an accurate determination of the amount of transcribable DNA in chromatin can be made. It should also be emphasized that the amount of transcribable DNA determined with this assay *in vitro* and with bacterial polymerase does not necessarily reveal how much DNA is actually transcribed *in vivo*, but it does detect changes which may occur in the state of the DNA in chromatin in response to growth hormone, e.g. the availability of the chromatin DNA to be transcribed by an exogenous polymerase. It is note-

Fig. 5. Kinetics of RNA synthesis *in vitro* with isolated chromatin and bacterial RNA polymerase

(a) The incorporation of labelled UMP into the acid-insoluble product for 10 min with various amounts of template [μ g of DNA (as chromatin)]; (b) the incorporation of labelled UMP into acid-insoluble product at various incubation times with 5 μ g of DNA (as chromatin) as a template. All assays were performed with 6–10 units of enzyme activity at 37°C (Burgess, 1969). See the Methods and Materials section for further details.

Fig. 6. Comparison of the template capacity of isolated chromatin and the endogenous RNA polymerase II activity (DNA-like RNA synthesis) after injection of growth hormone (5 mg/rat) into (a) sham-operated and (b) hypophysectomized/adrenalectomized rats

●, Template capacity; ○, endogenous RNA polymerase II activity. See the Methods and Materials section for details on the analysis of chromatin template capacity. Values are expressed as a percentage of control values obtained from similarly treated rats injected with vehicle only (the animals were killed at the same time-interval). The mean and range of three replicate determinations are given.

worthy, however, that the template capacity of the chromatin from chick oviduct was shown to correlate with the number of RNA chain-initiation sites (Schwartz *et al.*, 1975).

Since 95% of the chromatin DNA in a cell nucleus is extranucleolar, our chromatin represents primarily nucleoplasmic chromatin and therefore serves as the template in the cell nucleus for the polymerase II, the major nucleoplasmic polymerase. It is therefore appropriate to assume that the amount of DNA in chromatin which is available for transcription may represent the rate-limiting factor in the activity of the nucleoplasmic polymerase II in the cell nucleus.

Fig. 6 shows the effects of growth hormone on the ability of rat liver chromatin to serve as template for RNA synthesis. The activity of the nucleoplasmic polymerase II is also plotted for comparison. Fig. 6(a) shows the effects of growth hormone on the chromatin template capacity and polymerase II activity in sham-operated rats. At the early period after

growth-hormone administration (1–10h) the response of the polymerase activity is different from that of the template capacity of the chromatin. Both are affected by growth hormone but the fluctuations are in opposite directions. At the later periods (48 and 72h) the template capacity and polymerase activity show similar responses, with each approaching the control value by 72h. With the hypophysectomized/adrenalectomized rats (Fig. 6b), the chromatin template capacity and polymerase II activity behave similarly during early time-periods, both increasing after growth-hormone administration. During later periods (10–72h), both processes show little difference from control values. These results suggest that, at least in the case of hypophysectomized/adrenalectomized rats, the activating effects of growth hormone on the nucleoplasm polymerase II activity could be explained in terms of a derepression of the DNA template in the chromatin. With the sham-operated rats, however, this probably is not the case since the chromatin template capacity remains below control values from 1 to 48h whereas the polymerase II activity rises above control values from 6 to 10h.

Discussion

One aspect of this study which sets it apart from the majority of the other studies concerning growth-hormone effects on RNA synthesis is the characterization of the various products (RNA) synthesized *in vitro* by the nuclei in our assay conditions. The use of a specific inhibitor (α -amanitin) of the nucleoplasmic RNA polymerase (polymerase II) allows a more sensitive assay for the nucleolar RNA polymerase (polymerase I). In addition, the modified method of nuclei isolation and the elimination of detectable ribonuclease activity during the assays ensures a more accurate analysis of both types of polymerases. Although practically all hormones have some effect on the permeability of metabolites in cells (Hechter & Halkerston, 1964), our assays *in vitro* for the polymerases were performed with saturating amounts of nucleotides and the results should be independent of the effects of cell-membrane permeability or nucleotide pools. A rapid response of the polymerases to growth-hormone treatment therefore suggests a very important role for the transcription process in the mechanism of action of growth hormone. Such a role for the transcription process has been assigned to the action of steroid hormones (Hamilton, 1968; O'Malley *et al.*, 1969; Glasser *et al.*, 1972).

It is generally accepted that when hypophysectomized/adrenalectomized rats are given growth hormone the rate of RNA synthesis is accelerated, followed by that of protein synthesis and finally an induction of growth (Tata, 1970). This is substantiated in our studies by the growth-hormone activation in hypophysectomized/adrenalectomized rats of the nucleo-

lar polymerase I from 1 to 4h and the nucleoplasmic polymerase II from 1 to 10h. These activations precede the reported activation of protein synthesis. In our experiments the early enhancement by growth hormone of the nucleolar polymerase I which synthesizes ribosomal RNA correlates with a previously reported observation that growth hormone does activate ribosomal RNA synthesis and increases the hepatic polyribosomes in hypophysectomized/adrenalectomized rats (Tata, 1970). Our results concerning the growth-hormone effects on the nucleoplasmic polymerase II activity differ from those reported previously (Jänne & Raina, 1969; Smuckler & Tata, 1971).

However, activation of polymerase I as well as polymerase II activities within 1h after growth-hormone administration was reported by Salaman *et al.* (1972), supporting our results. In our studies the activation of polymerase II continues up to 8h, whereas Salaman *et al.* (1972) observed a decline to control values by 3–4h. The dose of active growth hormone may play a role in these differences.

Our studies also suggest that changes in the ability of the DNA to serve as a template for transcription appear to be at least partly involved in the growth-hormone alteration of RNA synthesis. It is noteworthy that the amounts of transcribable DNA in the liver chromatin in our studies did not follow in all instances the activity of polymerase II. This could be explained as follows: (1) both polymerase II and gene restriction are affected by growth-hormone treatment, or (2) the method of analysing the chromatin template capacity using a bacterial polymerase is not representative of the actual amount of DNA which is transcribed *in vivo*. Other workers have reported that growth hormone does not alter the template capacity of the chromatin in rat liver (Smuckler & Tata, 1971) or skeletal muscle (Breuer & Florini, 1966). There is no obvious explanation for this discrepancy. Our assays for template capacity were performed such that nucleotide incorporation was shown to be linear, and the amounts of template were shown to be rate-limiting, with no detectable RNAase activity present during the assay. Different methods of growth-hormone treatment as well as different conditions for analysis of the template capacity may possibly explain these discrepancies.

It is suspected that the RNA synthesized by the nucleoplasmic RNA polymerase II might also include the messenger RNA that is translated into enzymes such as the mixed-function oxidases. Indeed, Jacob *et al.* (1974) have reported that the specific inhibitor of RNA polymerase II, α -amanitin, prevents the phenobarbital induction of microsomal mixed-function oxidase activities. However, during the first 10h after growth-hormone treatment, the RNA polymerase II activity is activated in hypophysectomized/adrenalectomized rats, whereas the mixed-

function oxidase activities are decreased [see the preceding paper, Wilson & Spelsberg (1976)]. This is not unreasonable, since only a fraction of the DNA-like RNA synthesized by polymerase II leaves the nucleus (Harris, 1964; Attardi *et al.*, 1966; Scherrer & Marcaud, 1969). Further, only a small fraction of that RNA which does leave the nucleus is coded for these mixed-function oxidase enzymes. Consequently no correlation between polymerase II activity and that of the mixed-function oxidases should necessarily be observed, since the majority of the products of RNA polymerase II activity are assigned to other cell functions.

It is more than likely that transcription of messages for protein involved in cell growth rather than that involved in mixed-function oxidase activity, occurs during the early period (1–10h after growth-hormone treatment) of enhanced polymerase I and II activity. Indeed, Russell *et al.* (1970) demonstrated that ornithine decarboxylase activity in rat liver is enhanced within 2–3h after growth-hormone treatment and that this enhancement is based on early RNA synthesis. These data are consistent with the proposed hypothesis that mixed-function oxidase activity is decreased in situations associated with liver growth (primarily by mitogenesis) (Wilson & Frohman, 1974), as discussed in the preceding paper (Wilson & Spelsberg, 1976).

We do not understand the precise mechanism by which this hormone affects the transcription process, simply because we have little knowledge of the basic systems which regulate gene activity in eukaryotic cells. Other aspects of gene expression, such as mRNA stability, efficiency of translation, and protein turnover rates, may be (and probably are) affected by growth hormone and deserve investigation.

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