Effect of Growth Hormone on Ribonucleic Acid Metabolism

THE TEMPLATE ACTIVITY OF THE CHROMATIN AND MOLECULAR SPECIES OF RIBONUCLEIC ACID SYNTHESIZED AFTER TREATMENT WITH THE HORMONE

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Growth hormone stimulates the synthesis of RNA in hypophysectomized rat liver. The question whether the hormonal stimulation of RNA synthesis is due to the activation of repressed cistrons or to other factors was studied. Nuclear RNA from the livers of adult female hypophysectomized and growth-hormone-treated rats was examined for molecular homology by hybridization techniques: no new species of RNA were detected after hormone treatment. The template activity of the chromatin for RNA synthesis is also not increased by the action of growth hormone. Short- and long-pulse-labelling experiments demonstrate that the hormonal stimulation of RNA synthesis is most marked in experiments where the period of incorporation of radioactive precursors is limited to 1–2 hr. It is concluded that the hormone influences essentially the rate of RNA synthesis in these tissues.

Previous work with growth hormone and oestradiol has demonstrated that these hormones stimulate the synthesis of RNA in the target organs. The activation of RNA synthesis is one of the early events of the action of these hormones (Talwar, 1963; Ui & Mueller, 1963; Talwar, Gupta & Gros, 1964; Talwar *et al.* 1965; Widnell & Tata, 1966; Florini & Breuer, 1966; Means & Hamilton, 1966). The physiological and metabolic effects of these hormones on the affected tissue are prevented by actinomycin D (Talwar & Segal, 1963; Ui & Mueller, 1963; Talwar *et al.* 1964).

The manner in which the hormones bring about an activation of RNA synthesis is not known. There is evidence that about 80% of the genetic information is repressed in animal cells in the interphase state (Allfrey, Littau & Mirsky, 1963; Littau, Allfrey, Frenster & Mirsky, 1964). It can be postulated that hormones give rise to an increased RNA synthesis by inducing the transcription of repressed cistrons. Morphological observations on the giant chromosomes of some insects lend support to this hypothesis. In the last-instar larvae of *Chironomus tentans*, ecdysone, a steroid hormone secreted by the prothoracic glands of the insect, causes a 'puffing' of certain chromosomes (Clever & Karlson, 1960). According to Beerman (1952), the 'puff' is an active locus of the synthesis of RNA in the chromosomes. Quantita-

tive and kinetic data on the 'puffing' phenomenon show that 'puffing' phases alternate with phases of 'non-puffing' in a tissue, and also that the magnitude of expression of a gene (i.e. the size of the 'puff') varies within large limits (Clever, 1963). There are thus two possibilities; the hormones, either directly or indirectly, activate the transcription of 'new' (repressed) genes, or they increase the rate of biosynthetic activities associated with the already active genes. In the first case hormones would bring about a quantitative increase in the amount of RNA synthesized on DNA as a template, and also give rise to qualitative changes, with 'new' species of RNA. In the second case there would be only a quantitative increase in RNA and no 'new' kind of RNA would be synthesized.

The present paper attempts to answer this question for the action of pituitary growth hormone on the liver of hypophysectomized rats. As reported earlier, growth hormone causes an increase in the incorporation of radioactive precursors into liver RNA (Talwar, Panda, Sarin & Tolani, 1962). Short-pulse-labelling experiments show a stimulation of the nuclear fractions of RNA. Both the ribosomal- and the messenger-RNA fractions are stimulated by the hormone treatment (Talwar *et al.* 1964; Korner, 1964). The qualitative nature of RNA obtained from livers of hypophysectomized rats and from those of rats given growth hormone

was examined by hybridization with homologous DNA. The template activity of the chromatin was also assayed in the two cases.

MATERIALS AND METHODS

Materials. Bac-T-Flex membrane filters, type B-6 (lot no. 377/6267), were obtained from Carl Schleicher and Schuell Co., Keene, N.H., U.S.A. Ionagar no. 2 was a product of Oxoid Ltd., London, E.C. 4. Pancreatic deoxyribonuclease I, electrophoretically purified and ribonuclease-free, was a product of Worthington Biochemical Corp., Freehold, N.J., U.S.A., and ribonuclease (five times crystallized) was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Tris and sodium dodecyl sulphate were the products of Sigma Chemical Co., St Louis, Mo., U.S.A. Cetyltrimethylammonium bromide was a product of Imperial Chemical Industries Ltd., Alderley Park, Cheshire. All other reagents used were A.R. grade from British Drug Houses Ltd., Poole, Dorset, or E. Merck A.-G., Darmstadt, Germany.

Radioactive precursors. [32 P]Orthophosphate, carrier-free or in iso-osmotic phosphate buffer, pH 7.4 (specific radioactivity 1.4 mc/ml.), was supplied by the Atomic Energy Establishment, Trombay, India. It was injected intraperitoneally at a dose of 2 mc/rat. The rats were killed 2 hr. after the injection of the radioactive precursor.

[14 C]Orotic acid (1 mc/42 mg.) and [3 H]orotic acid (1 mc/0.8 mg.) were the products of New England Nuclear Corp., Boston, Mass., U.S.A.

Treatment with growth hormone. Purified N.I.H. growth hormone, ovine and bovine, was kindly supplied by Dr M. Graff and Dr A. E. Wilhelm, or 'international standard for growth hormone', by the National Institute for Medical Research, London, N.W. 7 (through the courtesy of Dr J. R. Tata). The hormone was dissolved in 0.9% NaCl with minimal addition of NaOH. The pH of the final solution was 7.5–8. The hormone was injected intraperitoneally at a dose of 0.5 mg./day for 8–10 days. Control animals received an equivalent volume of 0.9% NaCl.

Preparation of nuclear fraction. Liver was homogenized in 5 vol. of sucrose-TMK buffer (final concentrations: sucrose, 0.25M; tris-HCl buffer, pH 7.5, 50mM; KCl, 25mM; MgCl₂, 5mM) in a Potter-Elvehjem homogenizer fitted with a Teflon pestle; 10–15 strokes were used. The homogenate was passed through a double layer of muslin cloth and centrifuged at 700g for 10 min. The pellet was resuspended in 0.25M-sucrose-TMK buffer (one-fifth of the initial volume), mixed with 2 vol. (v/v) of 2.3M-sucrose-TMK and layered over 1 vol. of 2.3M-sucrose-TMK in swinging-bucket (SW25.1) tubes, as described by Blobel & Potter (1966). Centrifugation at 50000g for 90 min. gave a pellet of clean nuclei as examined microscopically.

Preparation of RNA. (a) Nuclear RNA. RNA was extracted essentially by the method of Shearer & McCarthy (1967), except that RNA was precipitated in the final step with 2M-potassium acetate-25% (v/v) ethanol (final concentrations). This step is helpful in the removal of oligonucleotides, as recommended by Hiatt (1962).

(b) Total liver RNA. The tissue was homogenized in 8–10 vol. (v/v) of a medium containing (final concentrations): tris-HCl buffer, 0.01M; NaCl, 0.14M; bentonite, 50 mg./100 ml.; sodium dodecyl sulphate, 2.5% (w/v). The pH of the medium was adjusted to 7.4. The homogenate

was stirred in the cold for 30 min. with an equal volume of water-saturated phenol containing 0.1% of 8-hydroxy-quinoline. The aqueous phase was collected and the inter-phase material was extracted once again with the above-mentioned buffer and phenol. The mixed aqueous extracts were given one more treatment with phenol and the nucleic acids were precipitated with 0.4M-NaCl (final concentration) and 2 vol. of ethanol. The precipitate was collected and washed with 70% (v/v) ethanol followed by 95% (v/v) ethanol. The RNA was separated from glycogen by extraction with 2-methoxyethanol (Ralph & Bellamy, 1964). RNA extracted in this step was precipitated with 0.5 vol. of aq. 1% (w/v) cetyltrimethylammonium bromide soln. The cetyltrimethylammonium bromide-precipitated nucleic acid was washed two or three times with 70% ethanol containing 0.1M-sodium acetate, and then dissolved in 0.1M-sodium acetate and precipitated by adding 0.1M-NaCl (final concentration) and 2 vol. of ethanol. The precipitate was dissolved in 0.01M-tris-HCl buffer, pH 7.4, containing 1mM-MgCl₂, and treated with pancreatic deoxyribonuclease (5 μ g./ml.) for 30 min. in the cold. Two more phenol treatments were given to eliminate the proteins. The oligonucleotides were removed by precipitating the RNA in the final step by adding an equal volume of 4M-potassiumacetate and then ethanol to a final concentration of 25%.

Preparation of DNA. DNA was prepared from rat liver by the method of Marmur (1961). An additional treatment with Pronase was introduced after the ribonuclease step.

Hybridization. The procedure followed was that of Gillespie & Spiegelman (1965).

Sedimentation profiles on sucrose density gradients. The RNA preparations were analysed on sucrose density gradients essentially by the procedure of Gros *et al.* (1961) with the difference that 3 ml. of 50% (w/v) sucrose was layered at the bottom of the tubes followed by 22 ml. of a linear gradient of 5–20% (w/v) sucrose.

Template activity of chromatin. The chromatin was prepared from the livers of hypophysectomized and growth-hormone-treated rats and its template activity assayed by the method of Marushige & Bonner (1966). RNA polymerase was prepared from *Escherichia coli* A-19 by the method of Chamberlin & Berg (1962) and purified to stage 4.

RESULTS

Hybridization of nuclear RNA from the livers of hypophysectomized and growth-hormone-treated rats. To assess the extent of molecular homology or heterology of the RNA synthesized in rat liver after treatment of the animals with growth hormone, the RNA obtained in each of the two cases was tested for its ability to compete with the other for hybridization to DNA. In view of the observations that all species of RNA in the nuclei are not represented in the cytoplasm (Shearer & McCarthy, 1967), the nuclear RNA preparations obtained from livers of hypophysectomized and growth-hormone-treated rats were examined. Fig. 1 shows the RNA hybridized in the two cases as a function of increasing concentrations of DNA. The method of Gillespie & Spiegelman (1965) was used for these hybridiza-

Fig. 1. Hybridization of nuclear RNA from livers of hypophysectomized and growth-hormone-treated female rats against homologous DNA. B-6 membrane filters were impregnated with increasing amounts of rat liver DNA, and incubated in $6\times$ SSC medium (0.9 M -sodium chloride- 0.09 M -sodium citrate, pH 7.0) with $32\mu\text{g.}$ of ^{32}P -labelled nuclear RNA from the hypophysectomized or the hormone-treated animals, by the method of Gillespie & Spiegelman (1965). The specific radioactivities of nuclear RNA from hypophysectomized and hormone-treated animals were 548 000 and 776 000 counts/min./mg. respectively. The amount of RNA hybridized is shown as radioactivity (counts/min.) (a) and as a percentage of the total used (b). ●, RNA from hypophysectomized rats; Δ, RNA from growth-hormone-treated rats.

Fig. 2. Competition by unlabelled nuclear RNA from livers of hypophysectomized and growth-hormone-treated animals for the hybridization of ^{32}P -labelled nuclear RNA from hormone-treated animals. B-6 membrane filters impregnated with $150\mu\text{g.}$ of DNA were incubated in $6\times$ SSC medium (see Fig. 1) with $10\mu\text{g.}$ of radioactive RNA (specific radioactivity 592 000 counts/min./mg.) and increasing amounts of non-radioactive nuclear RNA from livers of either control or hormone-treated rats. ●, RNA from hypophysectomized rats; Δ, RNA from growth-hormone-treated rats. The ordinate shows the radioactivity of the ^{32}P -labelled hormone-treated RNA hybridized in either case.

radioactive RNA obtained from the hormone-treated rat liver (Fig. 2).

Similar conclusions have been independently arrived at by Drews & Brawerman (1967). However, a report has appeared indicating that animal-cell RNA could give rise to non-specific hybrid-formations in competition experiments (Birnbom, Pène & Darnell, 1967).

The results from the experiments described above suggest that the growth hormone does not induce new species of RNA in adult female rats to the extent detectable by these methods. The stimulatory effect of the hormone on the synthesis of RNA may be primarily due to its influence on the rate of synthesis.

Template activity of the chromatin prepared from livers of hypophysectomized and hormone-treated rats. To confirm that the increase of RNA in the livers of rats after treatment with the growth hormone was not due to the activation of repressed cistrons, the template activity of the chromatin from livers of hypophysectomized and growth hormone-treated rats was assayed. The chromatin obtained by the procedure of Marushige & Bonner (1966) had a low endogenous RNA polymerase activity, which was of the same order in the chromatin from the livers of control and growth hormone-treated rats (Fig. 3). The template activity of the chromatin preparations was severalfold lower than the priming ability of purified liver DNA. The chromatin from livers of growth hormone-treated rats did not have a higher template activity than the chromatin from livers of

tion studies. RNA preparations from both sources gave similar saturation curves. They were also observed to compete in a parallel manner with the

Fig. 3. Template activity of chromatin from livers of hypophysectomized and growth-hormone-treated rats. Growth hormone was injected subcutaneously (0.5 mg./rat/day) for 8 days. The chromatin was prepared from the liver of the hormone-treated and control animals, and its template activity for the synthesis of RNA was assayed by the method of Marushige & Bonner (1966). Δ , Chromatin from growth-hormone-treated rats; $\circ$, chromatin from hypophysectomized control rats; $\blacksquare$, purified rat liver DNA as template. The broken lines indicate the endogenous RNA polymerase activity in the two chromatin preparations ($\blacktriangle$, chromatin from growth-hormone-treated rats; $\bullet$, chromatin from hypophysectomized control rats).

Fig. 4. Ribonuclease-type activity in chromatin from livers of hypophysectomized and growth-hormone-treated rats. Increasing amounts of the two chromatin preparations were incubated with 100 μ g. of ^{14}C -labelled rat liver RNA (25340 counts/min./mg.) under the conditions in which their template activity was assayed, except that the four nucleoside triphosphates were omitted. After incubation at 37° for 10 min., 3 ml. of 10% (w/v) trichloroacetic acid was added to each tube. The precipitates were collected on Millipore filters and washed with 5% (w/v) trichloroacetic acid. Δ , Chromatin from growth-hormone-treated rats; $\circ$, chromatin from hypophysectomized control rats.

hypophysectomized rats; in fact, the activity was somewhat lower. Similar observations were made by Breuer & Florini (1966). The absorption

Fig. 5. Liver RNA from hypophysectomized and growth-hormone-treated rats after labelling for 1 hr. and 18 hr. *in vivo*. Bovine growth hormone or 0.9% NaCl was injected into hypophysectomized rats (0.5 mg./rat/day) for 8 days. The last injection of growth hormone or 0.9% NaCl was given 4 hr. before the rats were killed. The rats each received an injection of [$5\text{-}^3\text{H}$]orotic acid (12 μC /100 g. body wt.) 18 hr. before and of [$6\text{-}^{14}\text{C}$]orotic acid (15 μC /100 g. body wt.) 1 hr. before they were killed. RNA obtained in the two cases was analysed on a linear gradient of sucrose (5–20%). (a) RNA from hypophysectomized rats (specific radioactivity 10570 and 43630 counts/min./mg. of RNA for ^{14}C and ^3H respectively); (b) RNA from growth-hormone-treated rats (specific radioactivity 16880 and 49110 counts/min./mg. of RNA for ^{14}C and ^3H respectively). $\circ$, $E_{260}^{1\text{cm}}$; $\blacktriangle$, ^{14}C radioactivity; $\bullet$, ^3H radioactivity.

maxima of the two chromatin preparations were measured. The chromatin preparations from the livers of both the hypophysectomized and the growth-hormone-treated rats had maxima at 257–258 $\text{m}\mu$, and the E_{257}/E_{275} ratios were of the same order. The slight difference in template activity therefore does not appear to be related to preparation artifacts.

The chromatin preparations were tested for the presence of catabolic enzyme activities. The chromatin from livers of growth-hormone-treated rats had a higher ribonuclease-type activity (Fig. 4). When the endogenous nuclease activity in the

chromatin from livers of growth-hormone-treated rats is taken into account, the two chromatin preparations have more or less the same priming ability. In any event administration of growth hormone does not increase the template activity of the chromatin of adult rat liver.

Influence of growth hormone on the rate of synthesis of RNA. The experiments described above demonstrate that the hormone influences directly or indirectly the rate of RNA synthesis. To gain further understanding of the types of RNA whose rate of synthesis may be affected by administration of growth hormone, RNA was prepared from the livers of hypophysectomized and growth-hormone-treated rats after a short (1 hr.) or a long (18 hr.) pulse of radioactive precursors. The incorporation of [6-¹⁴C]orotic acid into RNA was measured after 1 hr. and that of [5-³H]orotic acid after 18 hr. *in vivo*. The specific radioactivity of the ¹⁴C-labelled RNA (1 hr. pulse) was 10570 and 16880 counts/min./mg. of RNA for hypophysectomized and hormone-treated animals respectively. The specific radioactivity of ³H-labelled RNA (18 hr. pulse) was 43630 and 49110 counts/min./mg. of RNA for hypophysectomized and growth-hormone-treated animals respectively. The hormonal stimulation is more perceptible in the short-pulse-labelling experiments, i.e. on the rate of synthesis of rapidly labelled RNA. The RNA preparations from livers of hypophysectomized and growth-hormone-treated rats were also analysed on a linear gradient of sucrose (5–20%). Fig. 5 shows that in the hormone-treated rats there is a rapid labelling of all categories of RNA but more particularly the RNA of sedimentation coefficients higher than 4 s, i.e. the ribosomal 28 s and 18 s species and the RNA between 4 s and 18 s. The labelling patterns with [³H]orotic acid (18 hr. incorporation) are approximately identical in the two cases.

DISCUSSION

Growth hormone influences a number of metabolic activities in the cells (Knobil & Hotchkiss, 1964; Pastan, 1966). Among other activities it stimulates the protein-synthesizing ability of the cells, this being demonstrable both *in vivo* and *in vitro* (Russel, 1955; Korner, 1961*a,b*). Growth hormone also stimulates the synthesis of RNA in liver and in cardiac and skeletal muscle (Florini & Breuer, 1966). The synthesis of both ribosomal and messenger RNA is stimulated by the hormone treatment (Talwar *et al.* 1964; Korner, 1964).

The manner in which the hormone treatment influences RNA synthesis is not clear. The activity of RNA polymerase tested in intact nuclei is observed to be enhanced by about 35% as early as 1 hr. after administration of the hormone (Widnell &

Tata, 1966). Perfusion of the liver *in situ* with a medium containing growth hormone gives rise to a detectable increase in the incorporation of radioactive orotic acid into RNA after 30 min. (Jefferson & Korner, 1967). The activation of RNA synthesis could be due to several factors, among which may be the following: (i) the template activity of the chromatin in physical or functional terms; (ii) an activation or increase of the RNA polymerase; (iii) the availability of triphosphates of the four nucleosides, especially when any one of them is present in limiting concentrations; (iv) an improvement in the efficiency of the transcription process by a combination of any of the above or other undefined factors (the recent reports on a factor implicit in the binding of messenger RNA to ribosomes are noteworthy in this connexion; Revel & Gros, 1967; Maitra & Hurwitz, 1967).

The present results suggest that growth hormone does not increase the template activity of the chromatin. Its action in this respect differs from that of the steroid hormones testosterone and oestradiol, both of which are reported to increase the template activity of the chromatin in their target organs (Breuer & Florini, 1966; Barker & Warren, 1966). Growth hormone does not induce the transcription of new species of RNA in adult female rats to the extent detectable by the available methods. It influences primarily the rate of synthesis of the rapidly labelled RNA. Essentially its effect is akin to an improvement of the efficiency of the transcription process. There have been reports of the positive influence of growth hormone on the uptake of amino acids in rat diaphragm *in vitro* (Peckham & Knobil, 1965). The regulating role of amino acids in RNA synthesis of bacterial cells has also been reported (Gros & Gros, 1958; Shih, Eisenstadt & Lengyel, 1966). These and other aspects need further investigation for the clarification of the mode of action of this hormone.

The presence of a low ribonuclease-type activity in the chromatin preparations is a puzzling observation. The activity of this enzyme was higher in the chromatin obtained from the growth-hormone-treated animals. The tissues of hormone-treated rats synthesize RNA at a faster rate. The finding of a higher nuclease activity in these chromatin preparations is apparently anomalous. It is difficult to state at this stage whether this nuclease activity is a contaminant in the preparation of the chromatin or whether it has any biological significance. Mention may be made of the reports of the initial synthesis of large-size RNA aggregates in animal tissues (Houssais & Attardi, 1966; Scherrer, Marcaud, Zajdela, London & Gros, 1966). Little is known about the transformation or breakdown of these aggregates into the cytoplasmic fractions of RNA. It is also not clear whether these processes

can be rate-limiting steps in the synthesis of RNA in animal tissues.

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