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*MODIFICATION OF TADPOLE LIVER CHROMATIN BY THYROXINE TREATMENT**

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Administration of thyroxine to tadpoles induces precocious metamorphosis which is accompanied by a number of biochemical changes in the liver. Carbamyl phosphate synthetase is one of the enzymes induced early and ahead of gross morphological changes by the hormone treatment.[1] Experiments using both whole animals[2] and liver slices[3, 4] established that the increase in carbamyl phosphate synthetase activity is the result of *de novo* net synthesis of the enzyme. It has also been found that thyroxine treatment increases the rate of synthesis of various types of RNA in tadpole liver.[5–7]

Inhibition of new RNA synthesis with actinomycin D abolishes carbamyl phosphate synthetase induction,[8] suggesting that new RNA synthesis is required for the enzyme synthesis.

The increased rate of synthesis of both RNA and carbamyl phosphate synthetase, after thyroxine treatment, could be due either to an increase in the amount or activity of RNA polymerase, or to other effects such as the modification of the template efficiency of chromatin.

The experiments reported in this study deal with the preparation of chromatin from tadpole liver nuclei free of endogenous RNA polymerase activity. Thyroxine treatment of tadpoles was found to modify the template efficiency of chromatin preparations.

Materials and Methods.—Animals and thyroxine treatment: Tadpoles, *Rana catesbeiana*, weighing between 6 and 7 gm, were purchased from Lemberger Co., Oshkosh, Wisconsin. Thyroxine treatment was carried out at 25°C for 11 days as described by Paik and Cohen.[1]

Preparation of liver nuclei and chromatin: The livers were removed and washed with frog Ringer solution and then homogenized in 5 vol of 0.25 *M* sucrose solution in standard buffer (500 mM Tris pH 7.4, 5 mM $MgCl_2$, and 25 mM KCl). The homogenates were filtered through four layers of cheesecloth and centrifuged at 700 × *g* for 10 min. The sedimented crude nuclear fraction was resuspended in 3 ml of original homogenizing medium and layered over 5 ml of 0.34 *M* sucrose in standard buffer. The nuclear fraction was sedimented again by centrifugation at 700 × *g* for 6 min. The washing procedure was repeated twice again. The washed nuclear fractions were further purified by suspension in 4 ml of 2.4 *M* sucrose in standard buffer and centrifugation for 30 min at 25,000 rpm in a Spinco SW39 rotor. Pure nuclei, which sedimented to the bottom of the tube, were washed once again with the original homogenizing medium.

Chromatin was prepared by the method of Dingman and Sporn[9] with slight modification. After removing the traces of cytoplasmic material by washing as described, the nuclear fraction was homogenized in a volume of solution F (0.2 mM trisodium EDTA, pH 7.1), and dialyzed

overnight against 2 liters of solution F without removing the sedimentable material. After dialysis, the contents of the dialysis bag were centrifuged at 18,000 × *g* for 30 min. The clear supernatant chromatin solution was then dialyzed to equilibrium against 0.01 *M* Tris buffer, pH 8. Chromatin preparations thus prepared were used either immediately or stored at 0°C.

Deproteinization of chromatin: Protein was removed from the purified chromatin essentially by the method of Huang and Bonner,[10] except that the DNA pellet was dissolved in 0.01 *M* Tris buffer pH 8 and dialyzed overnight against a volume of the same buffer to remove traces of CsCl.

RNA polymerase and assay of activity: RNA polymerase was a gift from Dr. Diez Söll of the Enzyme Institute, University of Wisconsin, and had a specific activity of 2160 units per mg protein. RNA polymerase was assayed by the method of Chamberlin and Berg.[11] The reaction mixture (0.125 ml) contained: 5 μmoles of Tris buffer, pH 8, 0.5 μmole of $MgCl_2$, 0.25 μmole of $MnCl_2$, 1.5 μmole of β-mercaptoethanol, 0.1 μmole each of ATP, CTP, GTP, and UTP, desired amounts of chromatin, and 50 units of enzyme. CTP was tritium-labeled with a specific activity of 10 μc per μmole. After incubation at 37° for 10 min, the reaction mixture was inactivated with 1 ml of cold 10% trichloroacetic acid. The reaction mixture was poured onto a Millipore filter (0.45-μ pore size) and the collected precipitate washed five times successively with 5-ml portions of cold 5% trichloroacetic acid and three times with 5-ml portions of ethyl ether. The filter (containing the precipitate) was air-dried and counted in a Packard scintillation spectrometer by the method of Mehrotra and Khorana.[12]

Preparation of labeled liver RNA: Tadpoles were each injected intraperitoneally with 5 μc of orotic acid-6-C^{14} (30 μc/μmole) 8 hr prior to sacrificing. The livers were removed, washed once in frog Ringer solution, and RNA was prepared by a modification of the method of Nakagawa and Cohen.[7] The final RNA preparation was dissolved in a volume of 0.01 *M* Tris, pH 7.4, and treated with pancreatic DNase (10 μg/ml) for 10 min at 25°C. Traces of protein were then removed by extraction with 90% phenol containing 1% sodium lauryl sulfate. RNA was finally precipitated from the aqueous phase with ethyl alcohol after addition of NaCl as described.[7] Specific activity of the RNA preparation was 29,400 cpm per mg RNA.

Chemical composition of chromatin: Histone was extracted by the method of Dingman and Sporn,[9] and measured by the method of Lowry *et al.*[13] using crystalline bovine serum albumin as a standard. DNA was measured by the method of Dische[14] using calf thymus DNA as a standard. RNA was determined after mild alkaline hydrolysis of the chromatin and the hydrolysate was adjusted to pH 2 with HCl. One optical density unit (at 260 mμ) of the hydrolysate was found to correspond to 32.5 μg of RNA.[7] Residual protein was determined by solution in a volume of 1 *N* NaOH as described by Lowry *et al.*[12]

TABLE 1

RNASE ACTIVITY OF PURIFIED CHROMATIN

	Fraction of hydrolysis
Control chromatin	0.05
Thyroxine-treated chromatin	0.07

Reaction mixture contained standard reaction components minus RNA polymerase and nucleotide triphosphate. C^{14}-labeled RNA (80 μg, sp. activity 29,378 cpm/mg RNA) and chromatin corresponding to 75 μg of DNA per 0.125 ml reaction mixture were used. RNase activity is expressed as the fraction of initial radioactivity rendered acid-soluble after 10 min incubation at 37°C.

TABLE 2

EFFECT OF AMMONIUM SULFATE ON RNA POLYMERASE ACTIVITY OF TADPOLE LIVER NUCLEI

	ATP incorporated (μμmoles)
Control nuclei	11
Control nuclei + ammonium sulfate	52.1
Thyroxine-treated nuclei	27.8
Thyroxine-treated nuclei + ammonium sulfate	36.4

Reaction mixtures contained in 1 ml vol the following components in μmoles: 100 Tris, pH 8; 5 NaF; 70 KCl; 5 $MgCl_2$; 10 cysteine; 0.2 each of CTP, UTP, and GTP; 1 μc/0.02 μmole ATP-C^{14}; and 1 OD (at 650 mμ) unit of nuclei. Saturated ammonium sulfate solution (0.05 ml) was added per ml reaction mixture as indicated. Incubation was carried out for 10 min at 37°C. Trichloroacetic acid-insoluble labeled material was assayed by the method of Gorski.[23]

Results.—When purified preparations of chromatin were used as the template for RNA synthesis using *E. coli* RNA polymerase, the chromatin prepared from liver nuclei of thyroxine-treated animals had a template efficiency 20–50 per cent higher than that of chromatin prepared from untreated animals (Fig. 1). Each point on the curves represents an average value obtained with three different chromatin preparations, which were made from a pool of livers from seven to ten animals. Intrinsic RNA polymerase in both types of chromatin preparations were negligible and no significant difference between them was observed.

In order to eliminate the possibility that the observed difference in the template efficiency of the chromatins was due to differences in RNase activity, chromatin RNase activity was determined (Table 1). Under the experimental conditions used for assay of RNA polymerase activity, the RNase activity in both systems was low, and the absolute molar differences in RNA hydrolysis were too small (and in the wrong direction) to explain the effect of thyroxine on the basis of decreased destruction of RNA. Furthermore, it can be seen in Table 2 that the differences in template efficiency of the chromatins are not the result of chromosomal modification during preparation. The modification in "genetic make-up" or the nuclear change induced by thyroxine treatment is apparent in the purified nuclei before the extraction of the chromatins. As is shown in Table 2, there is a higher rate of RNA synthesis in nuclei prepared from thyroxine-treated tadpoles. It has been reported that the addition of ammonium sulfate to so-called "aggregate RNA polymerase," or to isolated nuclei, stimulated RNA synthesis.[15] Ammonium sulfate had a similar effect on tadpole nuclei. Addition of $(NH_4)_2SO_4$ to nuclei prepared from untreated tadpoles resulted in a threefold increase in RNA synthesis, whereas the addition of $(NH_4)_2SO_4$ to nuclei prepared from thyroxine-treated tadpoles resulted in a lesser stimulation of RNA synthesis.

The exact mechanism involved in this stimulation of RNA synthesis is still not clear. However, it was observed in a separate experiment that addition of $(NH_4)_2SO_4$ caused the dissociation of about 15–20 per cent of nuclear histones associated with chromatins. The details of this study will be reported elsewhere. In any event, it is clear that the nuclear changes induced by thyroxine treatment have occurred before the chromatin was isolated. This fact eliminates the possibility that the differences observed in template efficiency are introduced during the preparation of the chromatin.

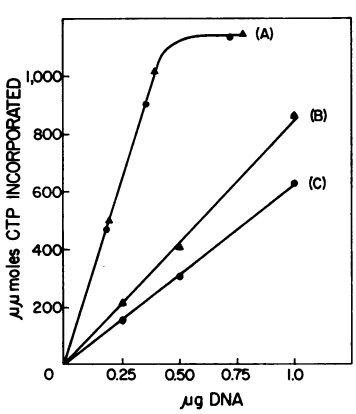

FIG. 1.—Experimental details are as given under *Methods*. Curve *A* represents RNA synthesis primed with deproteinized DNA. Curve *B*, ▲——▲ values with chromatin from thyroxine-treated tadpole liver. Curve *C*, ●——● values with control chromatin (no thyroxine).

When the chromatins prepared from both thyroxine-treated and control tadpoles were deproteinized with CsCl, the isolated DNA's showed equal template efficiency (Fig. 1). DNA thus prepared contained about 50 per cent of the original RNA and no detectable amount of protein. DNA obtained free from protein resulted in a fourfold increase of RNA synthesis. These experiments, together with the experiments involving $(NH_4)_2SO_4$ and whole nuclei (Table 2), suggest

TABLE 3

COMPOSITION OF PURIFIED CHROMATIN

Expt.	Samples	Histone/DNA	RNA/DNA	Residual protein/DNA
1	Control	0.77	0.017	0.18
	Thyroxine-treated	0.74	0.020	0.24
2	Control	0.71	0.015	0.25
	Thyroxine-treated	0.55	0.024	0.34
3	Control	0.91	0.023	0.22
	Thyroxine-treated	0.92	0.019	0.22

that the molecules involved in repression of genetic activity are proteins in nature. Similar conclusions have also been reached by other workers.[9, 16]

Examination of the chemical composition of chromatins indicated no significant differences in the RNA:DNA ratio and the protein:DNA ratio (Table 3). Slightly higher RNA:DNA and protein:DNA ratios have been observed in other systems where chromosome preparations were found to be active in RNA synthesis.[9, 17]

The preincubation of isolated chromatin with thyroxine gave no apparent modification of template activity in terms of RNA synthesis suggesting that other cellular mechanisms could be involved (Table 4). The chromatins prepared from nuclei preincubated with thyroxine *in vitro* did not show any better template efficiency (Table 5). In fact, it was observed that the treatment of nuclei with higher concentrations of thyroxine gave rise to chromatin preparations of slightly lower efficiency.

Discussion.—The administration of various hormones to rats has been reported to result in an increase in the liver aggregate RNA polymerase activity.[18–21] However, these nuclear enzymatic preparations were of such a nature that it has not been possible to establish whether the modification in the measured activity was the result of an increase in the amount of active RNA polymerase or to other factors such as template efficiency of DNA in the aggregate. Doly *et al.*[22] suggested that the increase in aggregate RNA polymerase of regenerating liver nuclei, or of prostate nuclei after androgen administration to castrated rats, was due to both a higher template efficiency and an increase in RNA polymerase activity. More recently, Dahmus and Bonner[16] reported that an increase in activity of a series of enzymes in the liver of rat following hydrocortisone treatment was the result of both modification of template and increased activity of RNA polymerase.

An enzymatically more active "aggregate RNA polymerase" can be obtained from

TABLE 4

EFFECT OF THYROXINE ADDITION ON CHROMATIN *in vitro*

Thyroxine	CTP incorporated ($\mu\mu$moles)
—	146
5.2×10^{-6} *M*	146
1.03×10^{-5}	134
2.06×10^{-5}	143
1.0×10^{-4}	142

Standard incubation mixture for RNA polymerase containing 0.25 μg of DNA in the form of chromatin from premetamorphic tadpole liver nuclei and 50 units of RNA polymerase; incubated for 10 min at 37°C.

TABLE 5

TEMPLATE EFFICIENCY OF CHROMATIN PREPARED FROM NUCLEI PRETREATED WITH THYROXINE

Thyroxine	CTP incorporated ($\mu\mu$moles)
Control	299
0.27×10^{-6} *M*	316
0.63×10^{-6} *M*	247
1.35×10^{-6} *M*	249

A suspension of approximately 9×10^5 nuclei per ml was treated with thyroxine for 10 min at 25°C before chromatin was extracted. Standard reaction mixture for RNA polymerase containing 0.12 μg DNA in the form of chromatin and 50 units of RNA polymerase were incubated for 10 min at 37°C. Values represent average of two experiments.

tadpole liver after thyroxine treatment. In the present studies, it has been shown that thyroxine modifies the chromatin so that it becomes an efficient template for RNA synthesis. It also appears that the protein moiety of the chromatin is more intimately associated with genetic repression. It has been suggested that nuclear polyanions, RNA, and protein may be involved in the regulation of genetic activity by their association with the nuclear histone fraction.[17]

Since the preincubation with thyroxine of both intact nuclei and chromatin gave no significant modification of the chromatin (i.e., its template efficiency), it is clear that these changes are brought about through more complex cellular mechanisms. Further studies are being made along these lines.

Summary.—It has been demonstrated that administration of thyroxine to tadpoles causes the modification of chromatin, prepared from liver nuclei, to a more efficient template for RNA synthesis. This modification of template efficiency is related to a protein moiety associated with chromatin.

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