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*INCREASED TEMPLATE ACTIVITY OF LIVER CHROMATIN, A RESULT OF HYDROCORTISONE ADMINISTRATION**

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We have found that the administration of hydrocortisone to adrenalectomized rats increases the template activity of their liver chromatin for RNA synthesis. Such administration is known to cause a two- to threefold increase in rate of nuclear RNA synthesis in the liver.[1, 2] This increase is followed by an increase in the activities of a series of liver enzymes.[3–5] Since the induction of these enzymes by hydrocortisone is abolished by simultaneous treatment with actinomycin D, it is clear that new RNA synthesis is required to support their formation.[4, 5] The increased rate of liver RNA synthesis caused by administration of hydrocortisone might in principle be due to changes in the template activity of the liver genetic material such as would accompany derepression of genes previously repressed. We shall show below that the administration of hydrocortisone does result in an increased availability of the genetic material for transcription.

Materials and Methods.—Treatment of rats: Male albino Sprague-Dawley rats weighing 150–250 gm each were obtained in the bilaterally adrenalectomized condition from Berkeley Pacific Laboratories. Hydrocortisone A grade was obtained from Calbiochem.

Rats were fasted 18 hr prior to the beginning of the experiment. Hydrocortisone-treated rats were given an intraperitoneal injection of hydrocortisone, 5 mg/100 gm body weight, suspended in physiological saline. Control rats were injected with an equal volume of saline. Four hours after treatment the rats were sacrificed, their livers immediately removed and washed with cold saline. The livers were then frozen in dry ice and chipped into small pieces. Tissue from identical treatments (24 rats per treatment) was pooled and stored at −80°C.

Preparation of purified chromatin: Crude chromatin was prepared from 10-gm samples of frozen tissue by the procedures of Marushige and Bonner[6] with the modification that the tissue was homogenized in 0.05 *M* NaCl plus 0.016 *M* Na_2 EDTA (pH 8.0). The chromatin was purified by centrifugation through 1.7 *M* sucrose and dialyzed against two changes of 0.01 *M* tris, pH 8.0.

Preparation of deproteinized DNA: Protein was removed from the purified chromatin by centrifugation in 4 *M* CsCl according to the method of Huang and Bonner.[7] Samples were centrifuged at 35,000 rpm for 22 hr in a Spinco SW-39 rotor. The gelatinous DNA pellet was dissolved in 0.01 *M* tris, pH 8.0.

Preparation of RNA polymerase: RNA polymerase was prepared from early log phase cells of *E. coli* strain B (General Biochemicals) by the methods of Chamberlin and Berg[8] to the stage of their fraction 3, hereafter referred to as F_3.

Assay of template activity: The complete incubation mixture for RNA synthesis contained in a final volume of 0.25 ml: 10 μmoles tris buffer (pH 8.0), 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmoles β-mercaptoethanol, 0.05 μmole spermidine phosphate, 0.10 μmole each of CTP, UTP, and GTP, 0.10 μmole 8-C^{14}-ATP (spec. act. 1 μc/μmole), DNA or chromatin, and F_3. Samples were incubated at 37°C for 10 min. The reaction was then stopped by the addition of cold 10%

TCA. Acid-insoluble material was collected by filtration on TCA-presoaked membrane filters (Schleicher and Schuell B-6) and washed with four 5-ml portions of cold 10% TCA. The filters were then glued to planchets, dried, and counted in a Nuclear-Chicago D-47 gas flow counting system. That the 8-C^{14}-ATP is in fact incorporated into RNA has been shown by Marushige and Bonner.[6]

Enzyme assays: Tyrosine transaminase was determined following the method of Canellakis.[9] An aliquot (10 ml) of the first homogenate in the preparation of chromatin was frozen and stored at −80°C. This freezing and storage for several days at −80°C resulted in no loss of enzyme activity. The samples were thawed, further homogenized with 0.05 ml 2-octanol in an Omni Mixer (75 v-75 sec), and filtered through one layer of Miracloth. Aliquots (0.1 ml) were used for enzyme assays, and 1-ml aliquots were used in the determination of protein.

The loss of TCA-precipitable material from C^{14}-labeled RNA brought about by incubation of C^{14} RNA with purified chromatin was taken as a measure of RNase activity.

C^{14}-labeled RNA was prepared by incubation of liver DNA in the medium for RNA synthesis as outlined above. The incubation mixture was then diluted with 0.01 *M* NaOAc, pH 5.5, containing 5% butanol, 1% sodium lauryl sulfate, 10^{-3} *M* $MgCl_2$, and treated with an equal volume of water-saturated phenol (60°C) for 3 min. Carrier RNA was added and RNA twice precipitated with ethanol. The final C^{14}-RNA pellet was dissolved in 0.01 *M* tris, pH 8.0 (spec. act. 95,000 cpm/mg RNA). C^{14}-labeled RNA was incubated with purified chromatin in the standard mixture for RNA synthesis minus F_3 and nucleoside triphosphates. After 10 min incubation at 37°C, C^{14}-labeled RNA was assayed as described above under assay of template activity.

Chemical composition: DNA was determined by the diphenylamine method of Dische[10] using rat liver DNA as a standard. RNA was determined by the orcinol reaction following the method of Dische and Schwarz[11] using purified yeast RNA as a standard. Histone was extracted from chromatin with 0.2 *N* H_2SO_4 at 4°C and precipitated with 20% TCA. Its amount was determined following the method of Lowry *et al.*[12] using rat liver histone as a standard. Nonhistone protein was determined on the acid-insoluble material by the same procedure, using bovine serum albumin fraction V as a standard.

Results.—A single intraperitoneal injection of hydrocortisone brings about an increase in the activity of liver tyrosine transaminase, as is presented in Table 1. This induction represents a five- to sixfold increase, indicating that our system is responding as previously reported.[3–5, 13]

Purified chromatin was prepared from livers of rats 4 hr after treatment either with hydrocortisone or with saline. The chromatin isolated from the livers of hydrocortisone-treated animals will be referred to as "induced" chromatin, while

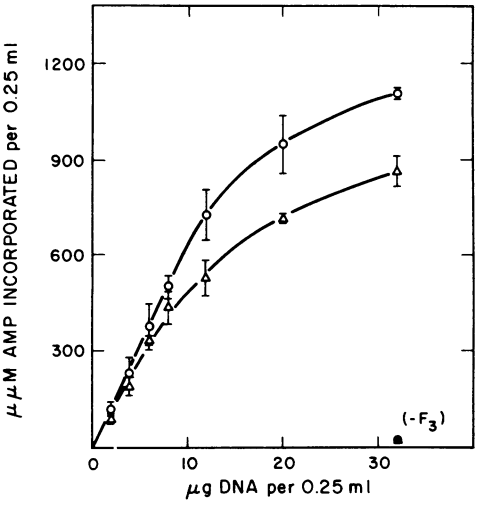

FIG. 1.—Template activity of rat liver chromatin isolated 4 hr after treatment with hydrocortisone (—O—O—) or saline (—Δ—Δ—). The incubation mixture (0.25 ml) contained various concentrations of DNA in the form of chromatin and 58 μg F_3. Incorporation by F_3 alone (100 μμM AMP/0.25 ml) has been subtracted. Each point is an average of three incubations. The brackets represent 90% confidence limits.

Samples incubated without added exogenous RNA polymerase are shown: (−F_3), ● = hydrocortisone-treated; ▲ = saline-treated.

TABLE 1

INDUCTION OF LIVER TYROSINE TRANSAMINASE BY HYDROCORTISONE TREATMENT OF ADRENALECTOMIZED RATS

Liver from:	Tyrosine transaminase activity* (μg product/30 min)
Control animals	13
Hydrocortisone-treated animals	72

* Activity is expressed as μg of p-hydroxy-phenylpyruvate formed per 30 min per mg protein at 38°C. Tyrosine transaminase was assayed 4 hr after treatment (see *Materials and Methods*).

TABLE 2

RNASE ACTIVITY OF PURIFIED LIVER CHROMATIN

Chromatin from livers of:	RNase activity*
Control animals	0.09
Hydrocortisone-treated animals	0.12

* The incubation mixture contained, in a final volume of 0.25 ml, 32 μg of purified chromatin and 25 μg C^{14}-labeled RNA in the standard mixture for RNA synthesis minus F_3 and nucleoside triphosphates. RNase activity is expressed as the fraction of initial C^{14}-labeled RNA (2,400 cpm) rendered TCA-soluble after a 10-min incubation with chromatin at 37°C. The values reported are the averages of two samples.

that isolated from saline-treated animals will be referred to as "noninduced."

The template activity for RNA synthesis of the two types of liver chromatin in the presence of added exogenous RNA polymerase is shown in Figure 1. It is clear that any given amount of DNA supplied as induced chromatin supports a greater rate of RNA synthesis than does an equal amount of DNA supplied as noninduced chromatin. The data of Figure 1 indicate that in the presence of added RNA polymerase at high template concentrations the template activity of induced liver chromatin is approximately 30 per cent greater than that of noninduced chromatin.

Chromatin purified according to the present procedure possesses essentially no endogenous RNA polymerase activity, as is apparent in Figure 1 ($-F_3$). No difference in the rate of RNA synthesis supported by induced chromatin or noninduced chromatin could be detected when such synthesis was catalyzed solely by endogenous polymerase.

The increase in template activity indicated in Figure 1 has been shown to be statistically significant by two methods. An analysis of variance shows that the increase of template activity induced by hydrocortisone is significant at the 99 per cent level. In addition, a regression analysis of the slopes of the linear portion of the template saturation curves shows that they are significantly different at the 95 per cent level. The increased template activity of induced chromatin is reproducible from experiment to experiment, ranging from 10 to 35 per cent.

The difference in template activity between induced and noninduced chromatin is maintained over the range of RNA polymerase concentrations 30–120 μg per 0.25 ml, and also over the range of nucleoside triphosphate concentrations 0.05–0.20 μmole each per 0.25 ml. The RNase activity of rat liver chromatin is small and not significantly different between induced and noninduced chromatin (Table 2). It seems, therefore, that the difference in rate of RNA synthesis observed is not due to the presence of degradative enzymes but rather to a real difference in template activity.

The difference in template activity between induced and noninduced chromatin is abolished by removal of DNA-bound protein. Purified liver chromatin was suspended in 4 *M* CsCl and centrifuged as described under *Methods* for the preparation of deproteinized liver chromatin DNA. The DNA thus isolated has an increased template activity of about 14-fold as compared to rat liver chromatin. The data of Figure 2 show that there is no difference in template activity between in-

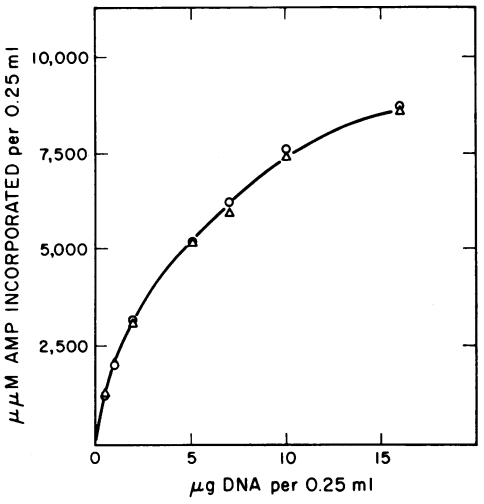

FIG. 2.—Template activity of CsCl deproteinized DNA. The incubation mixture contained various concentrations of deproteinized DNA and 58 μg F_3. Incorporation by F_3 alone (100 μμM AMP/0.25 ml) has been subtracted. —O—O—, Deproteinized DNA from induced chromatin of Fig. 1; —Δ—Δ—, deproteinized DNA from noninduced chromatin of Fig. 1.

duced and noninduced chromatin when the proteins associated with the DNA are removed. The DNA samples prepared from induced and noninduced chromatin are of similar molecular weight. Sedimentation coefficients were determined by boundary velocity sedimentation in the Spinco model E centrifuge and molecular weights calculated using the relation of Studier.[14]

The chemical compositions of induced and noninduced liver chromatin are given in Table 3. There is no detectable analytical difference in the amounts of the several components measured.

Addition of hydrocortisone to noninduced liver chromatin *in vitro* causes no increase in template activity for RNA synthesis. Preincubation of chromatin with hydrocortisone at 4° or at 37°C for 10 min prior to the addition of polymerase also elicits no effect upon liver chromatin template activity (Table 4).

Discussion.—The experiments reported above clearly demonstrate that the administration of hydrocortisone to adrenalectomized rats causes an increased template activity of liver chromatin for RNA synthesis. Lang and Sekerkis[15] and Barnabei *et al.*[16] have previously reported the isolation of template-polymerase omplexes from the liver of hydrocortisone-treated rats, and have shown that such

TABLE 3

CHEMICAL COMPOSITION OF RAT LIVER CHROMATIN

Component	Mass Ratios* Induced chromatin	Mass Ratios* Noninduced chromatin
DNA	1	1
RNA	0.059	0.056
Histone	0.82	0.82
Nonhistone protein	1.01	1.06

* Average values of four preparations.

TABLE 4

EFFECT OF HYDROCORTISONE ON TEMPLATE ACTIVITY OF PURIFIED CHROMATIN *in vitro*

μg Hydrocortisone/ 0.25 ml	μμM ATP incorporated/0.25 ml
0	561
10^{-10}	587
10^{-8}	587
10^{-6}	576
10^{-4}	575
10^{-2}	559
1	591
10	587

Each 0.25-ml incubation contained 26 μg DNA in the form of noninduced chromatin and 78 μg F_3. Hormone was preincubated 10 min at 4°C before the addition of F_3. Incorporation by F_3 alone (208 μμM AMP) has been subtracted.

complexes possess greater activity for RNA synthesis than do similar complexes from rats not treated with hydrocortisone. Their experiments do not, however, distinguish between increased rate of RNA synthesis resulting from increased template activity of chromatin and increased rate of RNA synthesis resulting from increased activity or amounts of RNA polymerase contained in the complex. In the present experiments the addition to the chromatins of equal and large amounts of exogenous RNA polymerase makes possible a clear measure of template activity.

The increase in template activity which results from hydrocortisone action would appear to be in some way associated with the proteins which are complexed with the chromosomal DNA. This follows from the fact that removal of chromosomal protein by treatment with high salt concentrations yields DNA of equal template activity from induced and noninduced liver chromatin. Differences in protein composition of chromatin induced by hydrocortisone treatment, if any, are apparently too small to be detected by present analytical methods.

The increase in rate of RNA synthesis by rat liver nuclei in response to hydrocortisone treatment is of the order of two- to threefold. The increases in template activity for RNA synthesis of rat liver chromatin caused by hydrocortisone treatment are of the order of 30 per cent. It is clear, therefore, that a portion of the increase in rate of RNA synthesis elicited by hydrocortisone administration is due to effects not preserved in isolated chromatin. A further portion may be due to an increased concentration or activity of RNA polymerase itself.

Finally, although the administration of hydrocortisone *in vivo* causes increased template activity to be developed *in vivo*, the administration of hydrocortisone directly to isolated, noninduced chromatin has no such effect. Therefore, there is some intermediary, not preserved in isolated chromatin, between the hormone and its ultimate effect upon the state of repression of the genetic material.

Summary.—Chromatin isolated from the liver of hydrocortisone-treated adrenalectomized rats possesses a greater template activity for DNA-dependent RNA synthesis than does chromatin isolated from the livers of rats not treated with hydrocortisone. This difference in template activity is abolished by the removal of proteins associated with the DNA.

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*INDUCED ORIENTATION OF THE GROWTH OF MALIGNANT CELLS IN VITRO**

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During the past $2^1/_2$ years we have studied the effect on malignant cell cultures of various growth-retarding and growth-promoting extracts. The extracts have been derived from sources such as calf thymus, human urine, and clams.

After examining about 43,000 cultures microscopically in the course of these studies, we noticed a peculiar appearance of the cells which had been treated with a particular extract. This extract had been prepared from human urine. Figure 1*A* shows the appearance of a 90-hr-old control culture of KB cells, while Figure 1*B* shows a parallel culture, exposed to the extract for a period of 66 hr. The agent caused the cells to become bipolar and orient themselves in straight rows. The chains of cells were usually parallel to each other and to the long axis of the coverslip on which they were grown. This parallel arrangement of the cells occurred equally well whether the cells were actually touching or situated at a distance from each other. The coverslips used for these experiments were new, unused, and had been subjected to an identical cleaning procedure. They were randomized, and none of the control cultures, nor parallel cultures treated with other urine extracts, showed any directional growth activity. The unknown agent was tentatively named directin, because of the way in which it can be identified.

The experiment was repeated with about 300 cultures, using extracts prepared from the original lot of urine, and from lots collected at different times during 1964 and 1965 at Otis Air Force Base. When the experiment was carried out with groups of 60 and 80 parallel KB-cell cultures exposed to an identical extract, directional growth was observed clearly in about 80 per cent of the cultures, while the remaining 20 per cent showed less striking results.

Preparation of the Extract from Human Urine.—About 1000 liters of urine were concentrated in a Turba film evaporator to 50 liters. The concentrate was kept at 4°C overnight, and the precipitate collected on a Sharples supercentrifuge. The precipitate was washed three times with 1 liter of distilled water and extracted with 2 liters of distilled water at pH 1–2. The acidic extract was precipitated with 6 vol of methanol and filtered. The filtrate was concentrated in a Buchler flash evaporator *in vacuo* to dryness. This dry material was dissolved in distilled water at pH 2 and filtered through 100 *g* Sephadex G-25 coarse gel at the same pH. Half-void volumes were collected. The directin was concentrated in V3/1 and there was also some activity in fraction V2/2.

Cell Culture Experiments.—The extracts were assayed on KB and HeLa (Flow