# TEMPLATE ACTIVITIES IN NORMAL, REGENERATING, AND DEVELOPING RAT LIVER CHROMATIN*

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Several hormones[1-5] have been shown to increase the ability of purified chromatin to mediate RNA synthesis in cell-free systems. In other systems not involving hormones, indirect evidence of changes in template activity has been forthcoming from studies utilizing endogenous RNA polymerase-DNA aggregates,[6] or intact nuclei isolated from normal and regenerating liver.[7] Direct proof is still lacking that the observed increase in RNA synthesis during active cellular growth involves modifications in template activity. We examined the template activity *in vitro* of highly purified chromatin from the liver of mature and developing rats, and from regenerating liver following partial hepatectomy. Template activity was found to change during development and during the first 20 hours of hepatic regeneration.

*Materials and Methods.*—Male Sprague-Dawley rats were used in all experiments, except those involving pregnant females. Weights varied according to age, the oldest animals weighing 250–300 gm. Partial hepatectomy, resulting in the removal of 68–72% of the entire liver, was performed by the method of Higgins and Anderson.[8] In one-day-old rats the operation was aided by gentle suction applied to the surface of the extruded liver while vascular and biliary connections were ligated. Sham-operated and unoperated animals were used as controls. For experiments on fetuses and newborn rats, pregnant females of known gestational age were obtained from the Charles River Labs. in Wilmington, Mass. These pregnancies were allowed to proceed to parturition, or were terminated on the 19th day of gestation.

*Preparation of chromatin:* The liver tissue was blotted on filter paper, weighed, washed in cold saline, and immediately frozen in dry ice. Two-gm samples of frozen tissue were used to prepare chromatin by the methods of Marushige and Bonner,[9] with the modification that the crude preparation was purified by centrifugation through 2.0 *M* sucrose at 50,000 *g* for 5 hr. The final solution of purified chromatin was examined spectrophotometrically for the presence of aggregates. Only solutions in which the ratio of optical density at 260/320 mμ was less than 0.05 were used in assays of template activity.

*Chemical composition of chromatin:* Protein was determined by the method of Lowry *et al.*[10] using crystalline bovine albumin as standard. DNA was analyzed by the diphenylamine reaction[11] with calf thymus DNA as standard and verified by measuring absorbance at 260 mμ. RNA was determined by the orcinol reaction[12] with purified yeast RNA as standard. Histones were extracted at 0° by suspension of the chromatin in 2 *M* NaCl for 1 hr. The solution was then made 0.2 *M* in HCl, stirred gently for an additional hour, and centrifuged at 20,000 *g* at 4° in the Spinco SW rotor for 20 min.[13] The supernatant solution was carefully removed with a Pasteur pipette, neutralized, and analyzed for histone protein. The pellet containing residual proteins was dissolved in 1 *N* NaOH in a boiling water bath and similarly analyzed for protein content. The sum of histone and residual proteins was always within 10% of the total protein values obtained with the original chromatin solution.

*RNA polymerase:* The enzyme was extracted from log-phase *Micrococcus lysodeikticus* by the procedure of Nakamoto *et al.*,[14] taken to fraction V. The purified polymerase was freshly prepared for each series of experiments, dissolved in 0.1 *M* Tris, pH 7.5, assayed for activity and DNA dependence, and stored in 50% glycerol at −20°C.

*Assay of template activity:* The reaction mixture for RNA synthesis contained in a total volume of 0.25 ml: 10 mμmoles Tris buffer (pH 8.0), 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmoles β-mercaptoethanol, 0.08 μmole each of ATP, CTP, UTP, and GTP, enzyme and chromatin or calf thymus DNA as template. Calf thymus DNA was used as a convenient measure of the

incorporating capacity of the polymerase in the reaction mixture after preliminary experiments had shown that deproteinized DNA[15] prepared from chromatin from normal or regenerating liver had similar template activities. ATP or UTP was labeled with $C^{14}$, specific activity 2.5 $\mu c/\mu$mole. Incubation proceeded for 30 min (the period of linearity with chromatin) at 37° and was terminated by the addition of 10% TCA. The acid-insoluble precipitate was washed twice with 1% TCA and twice with absolute ethanol. The material was treated overnight with solvent (supplied by Nuclear-Chicago, Inc.), 10 ml of toluene counting fluid was added, and the clear fluorescent solution was counted in a Packard scintillation spectrometer.

*Results.*—The template activity of liver chromatin, assayed in an *in vitro* RNA-synthesizing system with added bacterial polymerase, was significantly increased during regeneration. In a typical experiment with 150 $\mu$g of added polymerase (Fig. 1), chromatin from regenerating liver 20 hours after partial hepatectomy mediated the synthesis of approximately three times as much RNA as the chromatin from control liver. Both chromatin templates were far less efficient than native calf thymus DNA or protein-free DNA extracted from liver chromatin.

Changes in template efficiency during the first 24 hours of regeneration are shown in Figure 2 and Table 1, column 1. A large excess of enzyme and limiting amounts of DNA in chromatin were used in these experiments. In each case, chromatin containing 10 $\mu$g of DNA was incubated with 50 $\mu$g of *M. lysodeikticus* RNA polymerase. The amount of RNA produced on chromatin templates was constant during the first 4 hours after partial hepatectomy. Template activity then doubled between 4 and 6 hours after surgery. Activity remained unchanged

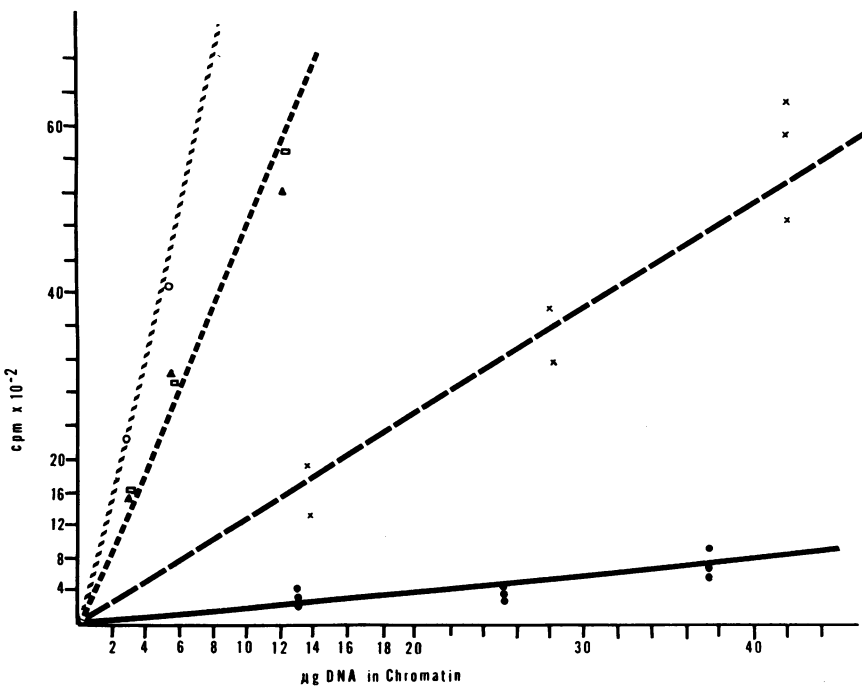


FIG. 1.—Template activity of calf thymus DNA (○), deproteinized DNA from normal (□) and regenerating liver (▲) and of chromatin from normal (●) and regenerating liver (x). Experimental details are given in the text. Incorporation by 150 $\mu$g of enzyme alone (473 cpm) has been subtracted. Each chromatin preparation was assayed at three dilutions, and each incubation was performed in triplicate.

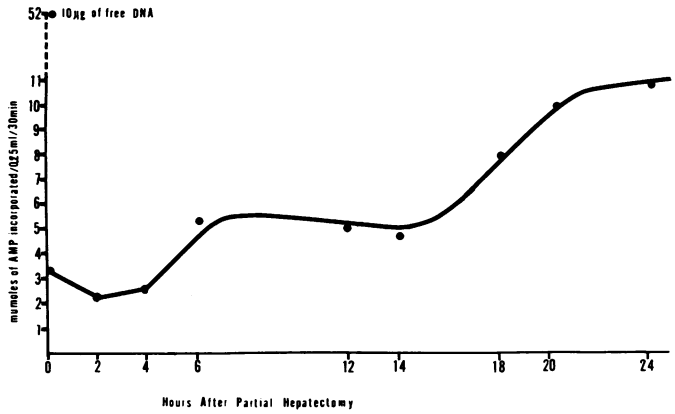


FIG. 2.—Template activity of chromatin during regeneration. The incubation mixture contained 10 μg of DNA in chromatin and 50 μg of enzyme. Incorporation by enzyme alone (0.183 mμM AMP) has been subtracted. Incorporation mediated by 10 μg of calf thymus DNA is also indicated. (Note change in scale.) Each point represents the average of results obtained with chromatin preparations from five animals, separately assayed in triplicate.

when tested at 12 and 14 hours of regenerative growth. Between 18 and 20 hours a further increase to more than three times the normal synthesizing capacity was noted. Template activity of pure DNA was 15-fold greater than chromatin from normal liver and 5-fold greater than the most active chromatin from regenerating liver.

A similar experiment with chromatin from liver at various stages in development is illustrated in Figure 3 and Table 1, column 1. The template capacity of the 19-day-old fetus was approximately 60 per cent of adult values. An increase to 145 per cent of the adult value occurred at the time of birth. This decreased to the adult value during the first 24 hours of postnatal life, and the livers of rats one or more days old had essentially similar template activities. Template activity in the livers of pregnant animals was lower than in newborn rats, but higher than in the livers of nonpregnant females and mature males (Table 1).

The composition of chromatin from normal, regenerating, and developing liver was generally similar with respect to the components analyzed (Table 2). The

TABLE 1

| Chromatin source | RNA Synthesis (cpm) Polymerase added (50 μg) | No polymerase added | Per cent |
|---|---|---|---|
| Normal adult liver | 3,000 | 250 | 8.3 |
| Pregnant female | 3,900 | 310 | 8.0 |
| Regenerating liver | | | |
| 2 hours | 2,740 | 190 | 7.0 |
| 4 hours | 2,980 | 208 | 7.0 |
| 6 hours | 5,810 | 294 | 5.0 |
| 12 hours | 5,660 | 247 | 4.4 |
| 20 hours | 11,435 | 433 | 3.8 |
| Developing liver | | | |
| 19-day fetus | 1,820 | 300 | 16.5 |
| Newborn rat | 4,970 | 430 | 8.7 |
| 1-day-old rat | 2,775 | 380 | 13.7 |
| Regenerating 1-day rat liver (6 hr) | 3,860 | 420 | 11.0 |

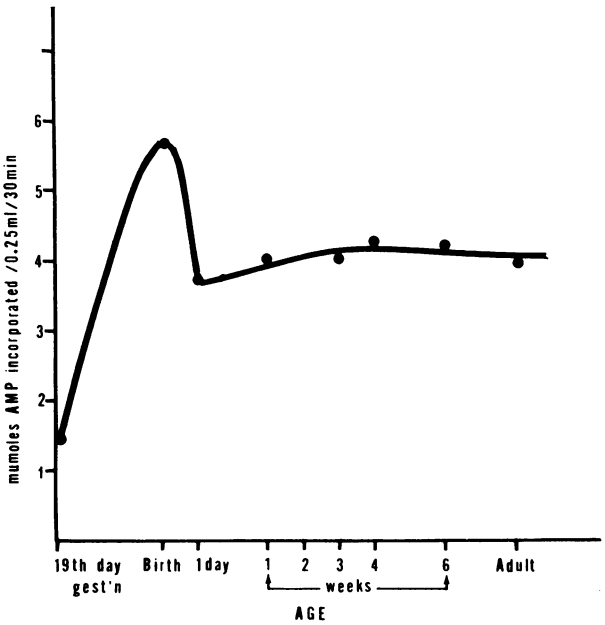


FIG. 3.—Template activity of chromatin during development. Experimental details as given in legend to Fig. 2. Points for fetal, newborn, and 1-day-old animals represent the average of results obtained with five individual chromatin preparations made by pooling 20 livers; points for older animals represent average results obtained with chromatin preparations from five animals, separately assayed in triplicate.

RNA content was generally $^1/_{20}$th of the DNA, the average total protein mass was 1.5 times that of DNA, and the binding of histone to DNA approached unity. The chromatin of partially hepatectomized one-day-old rats showed a diminished total protein content which was statistically significant.

Endogenous polymerase activity which remained bound to chromatin from normal, regenerating, and developing liver usually accounted for less than 10 per cent of the RNA synthesized in the presence of added bacterial polymerase (Table 1). This remnant of intrinsic activity which survived the purification procedures had no significant influence on the changes in RNA-synthesizing capacity of chromatin templates observed during the course of regeneration and development.

TABLE 2

COMPOSITION OF RAT LIVER CHROMATIN*

| Chromatin source | Mass Ratios DNA | RNA | Total protein | Histone |
|---|---|---|---|---|
| Adult liver | 1.0 | 0.052 | 1.73 ± 0.15 | 0.97 ± 0.10 |
| Regenerating liver | | | | |
| 2 hours | 1.0 | 0.041 | 1.43 ± 0.21 | 0.83 ± 0.16 |
| 4 hours | 1.0 | 0.049 | 1.35 ± 0.27 | 0.64 ± 0.22 |
| 6 hours | 1.0 | 0.028 | 1.57 ± 0.18 | 0.87 ± 0.13 |
| 12 hours | 1.0 | 0.046 | 1.49 ± 0.22 | 0.71 ± 0.18 |
| 20 hours | 1.0 | 0.033 | 1.32 ± 0.30 | 0.67 ± 0.12 |
| Developing liver | | | | |
| 19-day fetus | 1.0 | 0.058 | 1.63 ± 0.09 | 1.04 ± 0.14 |
| Newborn rat | 1.0 | 0.051 | 1.78 ± 0.18 | 0.83 ± 0.13 |
| 1-day-old rat | 1.0 | 0.048 | 2.31 ± 0.62 | 1.00 ± 0.18 |
| Regenerating 1-day-old rat | 1.0 | 0.036 | 1.06 ± 0.10 | 0.65 ± 0.40 |
| 1-week-old rat | 1.0 | 0.045 | 1.30 ± 0.25 | 0.69 ± 0.27 |
| 2-week-old rat | 1.0 | 0.049 | 1.13 ± 0.29 | — |
| 3-week-old rat | 1.0 | 0.056 | 1.30 ± 0.18 | 0.95 ± 0.15 |
| 4-week-old rat | 1.0 | 0.039 | 1.62 ± 0.30 | — |
| 6-week-old rat | 1.0 | 0.047 | 1.16 ± 0.53 | 0.87 ± 0.16 |

* Amounts of the components are expressed in relati on to the amount of DNA in the same sample of chromatin. Average of five preparations.

The ribonuclease activity of chromatin was evaluated in two ways: (1) Actinomycin D (10 $\mu$g) was added to the standard reaction mixture after 30 minutes of incubation, followed by further incubation. With all types of chromatin, an additional 8–12 per cent of radioactivity was incorporated into acid-insoluble product in the presence of inhibitor. (2) To eliminate the possibility that the RNA synthesized *in vitro* had already been hydrolyzed to RNase-resistant pieces at the time of addition of actinomycin D, $P^{32}$-labeled RNA (the gift of Dr. T. Fujii) was incubated with chromatin in the absence of enzyme and nucleoside triphosphates. RNase activity in all systems assayed was low, and insufficient to explain observed changes in template activity on the basis of decreased destruction of RNA (Table 3). It can be concluded that the increases in the rate of RNA synthesis observed in regenerating and developing liver chromatin are not due to endogenous "aggregate" RNA polymerase, nor differential RNase activity, but represent a real change in template efficiency.

TABLE 3

| Chromatin source | RNase activity |
|---|---|
| Normal adult | 0.08 |
| 6-hour regenerating | 0.06 |
| 12-hour regenerating | 0.11 |
| 20-hour regenerating | 0.09 |
| 19-day-old fetus | 0.12 |
| Newborn | 0.10 |
| 1-day old | 0.12 |

Reaction mixture contained standard components less enzyme and nucleotide triphosphates. $P^{32}$-labeled RNA (40 $\mu$g, sp. act. 12,263 cpm/mg) and chromatin containing 10 $\mu$g DNA were added in a total incubation volume of 0.25 ml. RNase activity is expressed as the fraction rendered acid-soluble after a 30-min incubation at 37°.

It has been suggested that spermidine plays a role in RNA production during hepatic regeneration.[16, 17] Its stimulatory effect on RNA polymerase activity has been repeatedly demonstrated.[18, 19] For these reasons, spermidine was excluded from our standard assays of template activity. Spermidine added to the incubation mixture had no significant effect in concentrations up to 2 mM and was inhibitory to all types of chromatin at higher concentrations.

It has been reported that ammonium sulfate exerts different effects on RNA synthesis in normal and regenerating liver when tested with RNA polymerase-DNA aggregates[6] or isolated intact nuclei.[7] Ammonium sulfate stimulated the reaction slightly (10–20%) at concentrations between 0.5 and 1.0 molar, had no effect at lower, and strongly inhibited (80–95%) the reaction at higher concentrations. Similar effects were observed when chromatins from normal, regenerating, or developing livers were used as template.

*Discussion.*—We have found that the template activity of chromatin from regenerating liver is tripled during the period in which cells are preparing for division. This increase can be divided into three distinct phases: template activity is doubled 4 to 6 hours after partial hepatectomy, remains constant between 6 and 12 hours, and is tripled between 14 and 20 hours. Such changes in template activity coincide with the pattern of RNA synthesis in the regenerating liver[20–22] and precede the rise of endogenous RNA polymerase at 12 to 18 hours[23–25] after partial hepatectomy.

The doubling of RNA synthesis *in vivo* during the initial 6 hours coincides with template changes which double their capacity to mediate this synthesis, without an appreciable increase in RNA polymerase activity. Between 6 and 14 hours the rate of RNA synthesis and template capacity remains unaltered, while polymerase activity rises to twice normal levels. These findings suggest that during the first

12 to 14 hours after partial hepatectomy, transcription from DNA is limited by the template.

There is an abrupt drop in the ratio of histone to DNA from 2.64 to about 1.0 during the first 8 hours after partial hepatectomy.[26] Fujioka *et al.*[20] have found a three- to fivefold increase in the free lysine pool of the liver 6 hours after partial hepatectomy, which returned to normal by 12 hours. This pattern was unaffected by inhibitors of RNA and protein synthesis. Thus, the initial stimulus for regeneration may involve the degradation of lysine-rich histone in the chromatin, thereby increasing its template capacity for the synthesis of RNA by "aggregate" RNA polymerase. This enzyme-DNA complex is activated by the removal of inhibitory histones.[27] The second increase in chromatin template activity, which occurs at 16 to 20 hours of regeneration and is accompanied by a similar stimulation of RNA synthesis *in vivo*, is complicated by the advent of active DNA[28] and histone synthesis.[26, 29] At this time, the free lysine pool remains unchanged, the amino acid being utilized for the production of histones coupled to the newly synthesized DNA. We have recently demonstrated a rapid turnover of lysine-rich histones in the nuclei of regenerating liver during the period of DNA synthesis.[30] However, the possibility that the newly replicated DNA in euchromatin is itself highly active as template when challenged with bacterial RNA polymerase has not been excluded.

The timing of these template changes is of interest in the light of recent data on the kinds of RNA produced during regeneration. Church and McCarthy[31] have shown that new kinds of messenger RNA are produced during the first 6 hours after partial hepatectomy in mice. New sites are therefore made available for copying and this is reflected in increased template activity during this period. By 12 hours, the number of new messenger species drops significantly. The period between 6 and 20 hours was not investigated by them. The functional significance of the stepwise increase in template activity during the transition from the normal to a regenerative state is clarified by the observation that low-level X-ray irradiation 6 hours after partial hepatectomy inhibited the formation of enzymes necessary for the synthesis of DNA which was to follow 12 hours later, whereas higher doses at 16 hours prevented DNA replication[32] and mitosis.[33] Tobey *et al.*[34] have produced evidence that specific RNA's are associated with cell division. The increase in template activity before mitosis begins in regenerating liver may represent the activation of sites required for this process.

Chromatin template activity during embryonic growth is lower than in postnatal liver. The fetal liver contains many cells in mitosis as well as a large proportion of hemopoietic elements. Both types of cell produce a smaller variety of RNA's than fully functional liver cells. Template efficiency is therefore lower in the fetus than in the adult, though more template is available in fetal liver because of high concentration of DNA.[35]

The perinatal period is characterized by a considerable increase in template activity followed by a lesser decline to adult levels. The rapidity and scope of these changes suggest release from inhibition, perhaps in combination with hormonal influences. The latter may also be responsible for the increased activity of liver chromatin in pregnant rats. Cortisol, which stimulates template activity[1] and causes the appearance of new species of messenger RNA in normal and regenerating liver,[16] induces enzymes under its control at birth, but cannot exert this effect

*in utero*.[37] The increase in template activity may therefore be due to the transcription of sites previously repressed. A variety of other enzymes also increase markedly at birth.[37] Thus, the increased capacity of chromatin for priming the synthesis of RNA is a reflection of accelerated differentiation as the liver acquires mature functions.

Partial hepatectomy at one day of age resulted in increased template activity, but elicited a smaller response after 6 hours of regeneration than in the adult. This may indicate that sites copied in the course of regenerative growth are shared by developmental growth processes to a larger extent than by the normal fully differentiated state.

Extensive alterations of chromatin template activity are not reflected by a corresponding pattern of changes in *gross* composition. Polyamines and high salt concentrations do not seem to play a significant role. However, when developmental and regenerative growth stimuli are combined, protein appears to be lost from chromatin. It may be concluded that control of genetic activity involves changes in chromosomal components, but current analytical methods are capable of detecting these changes only in the most extreme situations.

Abbreviations used: ATP, CTP, GTP, and UTP, the 5′-triphosphates of adenosine, cytidine, guanosine, and uridine, respectively; TCA, trichloroacetic acid.

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