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A Partial Sequence of Nuclear Events in Regenerating Rat Liver

(gene regulation/chromosomal RNA)

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ABSTRACT When two lobes of the liver of the adult rat are removed, the cells of the remaining lobe are aroused to renewed cell division. We have studied the early events in such regeneration. The first observable response to such partial hepatectomy is the production in the liver nuclei of rapidly-labeled high-molecular-weight RNA of sequences not produced by normal liver. This is followed, with a lag of about 1 hr, by the appearance of increased (above normal) amounts of chromosomal RNA, again of sequences not produced by normal liver. With a lag of another hour, the template activity in support of RNA synthesis of the liver chromatin increases substantially. These events occur before initiation of DNA synthesis in the cells of the regenerating liver.

Chromosomal RNA (cRNA) is a small nuclear RNA that comprises a significant fraction of the total nuclear RNA in all higher eukaryotes investigated (1–3). It hybridizes to the repetitive DNA sequences (4, 5) and is distinguished by a high content of dihydropyrimidine (6). Several experiments have suggested that cRNA is involved in the processes of gene activation (7, 3). In addition, it has recently been shown that cRNA and high-molecular-weight rapidly-labeled nuclear RNA (Hn RNA) share a large proportion of their nucleotide sequences, a result that strongly suggests a precursor–product relationship between them (5). To shed further light on these matters, we have investigated the temporal relationships between Hn RNA, cRNA, and template activity in regenerating rat liver.

MATERIALS AND METHODS

Regenerating Livers. The livers of 200–400 g male, albino Sprague-Dawley rats were caused to regenerate by surgically removing their two largest lobes (9). All operations were performed on rats anesthetized with ether, and timed so that the regenerating livers were harvested between 6 p.m. and 10 p.m. to minimize circadian effects. The rats were decapitated and their livers were quickly frozen in liquid nitrogen.

Chromatin. 20–40 g of frozen livers in saline–EDTA buffer (75 mM NaCl–24 mM EDTA, pH 8) were homogenized in a Waring blendor (85 V for 10 sec, 30 V for 3 min). This solution was then filtered through Miracloth (Chicopee Manufacturing Co., Milltown, N.J.) and centrifuged at 3500 rpm in the Sorvall centrifuge (SS-34 rotor) for 10 min. Crude chromatin was made from the pellet by three or four cycles of glass–teflon homogenization and centrifugation at 10,000 rpm (SS-34 rotor) for 10 min in 0.01 M Tris buffer (pH 8) (8). Chromatin for the template assays was purified by centrifugation through 1.7 M sucrose. The pellet was washed once by resuspension in 0.01 M Tris buffer (pH 8) and centrifugation at 10,000 rpm for 10 min. It was then sheared in a Virtis homogenizer at 45 V for 90 sec at a concentration of 10 A_{260}/ml. This solution was centrifuged for 30 min at 10,000 rpm in the Sorvall centrifuge and the supernatant was carefully removed and frozen in liquid nitrogen (8).

Preparation of Chromosomal RNA. Chromosomal RNA was prepared by one of two previously described methods. In the first, crude chromatin was dissociated in 4 M CsCl–0.01 M Tris (pH 8) and centrifuged at 36,000 rpm in the Spinco 40 rotor for 12–21 hr. Protein floats to the top to form a pellicle or skin. cRNA was obtained from the protein pellicle by digestion with Pronase, extraction with phenol, and elution from DEAE-Sephadex A-25 with a 0.2–1 M linear gradient of NaCl in 7 M urea. Chromosomal RNA elutes as a symmetrical sharp peak at 0.55 M NaCl (2). This method works erratically with regenerating livers; we have, therefore, adopted a new procedure that yields consistent results.

Crude chromatin was diluted with an equal volume of 1.7 M sucrose (enzyme grade, Mann Research), and the mixture was homogenized to a uniform consistency in a glass–teflon homogenizer. Then, with constant stirring, 0.3 M sodium deoxycholate was added dropwise to a final concentration of 15 mM, and the mixture was stirred for an additional 30 min at 4°C. It was then centrifuged at 36,000 rpm for 6–12 hr in the Spinco 40 rotor. The supernatant was decanted and made 7 M in urea and 0.2 M in NaCl by the addition of dry urea and NaCl. DEAE-Sephadex, which had first been equilibrated with 7 M urea–0.2 M NaCl, was then stirred in and a 0.9 × 20-cm column was poured with the slurry. When the column was formed and all the liquid had passed through, the cRNA was eluted with a gradient from 0.2 to 1.0 M NaCl in 7 M urea. As with the previous method, the cRNA elutes in a homogeneous peak at 0.55 M NaCl. The RNA was routinely eluted a second time from DEAE-Sephadex to remove residual impurities. All cRNA prepared by this method that was to be used for RNA–DNA hybridization on filters was then treated with Pronase, extracted with phenol, and precipitated with ethanol. If the RNA was not treated with Pronase, it bound nonspecifically to the nitrocellulose filters during the hybridization reaction, presumably because of the small amount of protein associated with it (3).

Abbreviations: cRNA, chromosomal RNA; SSC, standard saline citrate (0.15 M NaCl–0.015 M Na citrate)

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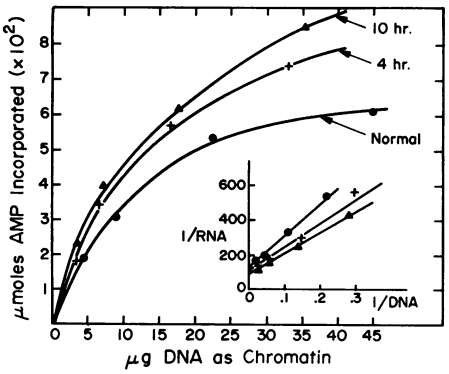

FIG. 1. Effect of template concentration on the rate of RNA synthesis directed by normal and regenerating rat-liver chromatin. The reaction rate at saturating amount of template (V_m) is calculated from a double-reciprocal plot, as shown in the inset.

In Vitro Labeling of cRNA. Chromosomal RNA was labeled *in vitro* by the addition of 1 mCi of [^{3}H]dimethylsulfate (New England Nuclear Corp., 100–900 Ci/mol) to 1 mg or less of cRNA in 12 ml of sodium phosphate buffer (pH 7.6), made with glass-distilled water (10). Incubation was for 6–12 hr at room temperature, and the RNA was recovered by ethanol precipitation and passage through a 2.5 × 30 cm Sephadex G-25 column.

RNA–DNA Hybridization. Denatured DNA was immobilized on nitrocellulose filters (Schleicher and Schuell, B-6, 12 mm) as described by Gillespie and Spiegelman (12). 10 μg of ^{14}C-labeled rat DNA was applied to each filter in 6× standard saline–citrate (6 × SSC; SSC is 0.15 M NaCl–0.015 M sodium citrate). Retention was normally about 70%.

Hybridizations were done at 37°C in 50% formamide and 5 × SSC, about 22°C below the melting temperature of native rat DNA in this solution (11). Each reaction was done in 0.2 ml of RNA solution, which contained two DNA filters and one blank filter in each reaction vial. At the end of the desired incubation time, the filters were removed, washed in 2 × SSC, treated with RNase (boiled pancreatic-ribonuclease A), washed again in 2 × SSC, dried, and counted in a Beckman liquid scintillation spectrometer.

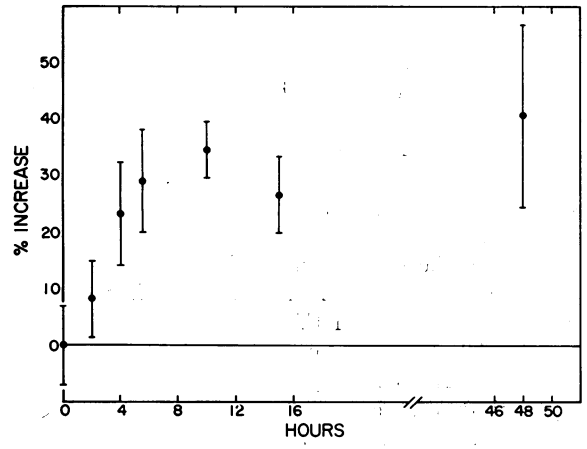

FIG. 2. % Increase in V_m values for transcription of regenerating-liver chromatin at various times after partial hepatectomy. *Error bars* represent the standard deviation of the data.

TABLE 1. *Template activities and % hybridization to denatured DNA of RNA transcribed from different chromatins*

Tissue source of chromatin	% of DNA hybridized by transcribed RNA	Template activity of chromatin compared to that of pure DNA (%)
Rat liver	6.1*	20
Rat kidney	3.1*	10
Pea bud	2.5†	5‡
Pea cotyledon	9.0§	30‡
Calf thymus	4.0¶	16

* Tan and Miyagi (18).
† (unpublished data).
‡ Bonner, *et al.* (6).
§ Bekhor *et al.* (7).
¶ Paul and Gilmour (reanalyzed) (16).

Preparation of RNA Polymerase. RNA polymerase was prepared from early-log-phase cells of *Escherichia coli*, strain B. Polymerase was purified by the method of Chamberlin and Berg (13), up to their fraction 4 (F_4). This material was dialyzed into buffer 1 [0.01 M Tris (pH 8)–0.5 mM dithiothreitol–0.1 mM EDTA–25% glycerol–0.15 M KCl], and run onto a 2 × 20 cm DNA–cellulose column (14). The polymerase was then eluted with a linear gradient from 0.15 to 1.0 M KCl in buffer 1. Nearly pure polymerase elutes at about 0.45 M KCl. Polymerase was stored frozen in liquid nitrogen.

The Template Assays. The complete incubation mixture for RNA synthesis contained, per 0.25 ml: 10 μmol Tris buffer (pH 8), 1 μmol $MgCl_2$, 0.25 μmol $MnCl_2$, 3 μmol 2-mercaptoethanol, 0.1 μmol of [^{14}C]ATP (2 Ci/mol), 0.1 μmol (each) of GTP, CTP, and UTP, chromatin, and RNA polymerase. Incubations were at 37°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 2 M NaCl, followed by a large excess of 10% trichloroacetic acid. The acid-insoluble material was collected by filtration through a nitrocellulose filter, which was then dried and counted in a Beckman Liquid Scintillation Spectrometer.

Rot. The term Rot has been introduced for RNA–DNA hybridization reactions where RNA is present in great excess. The term is strictly analogous to the well-established Cot, which has been defined for DNA–DNA hybridization reactions by Britten and Kohne (15). Rot stands for concentration of RNA nucleotides (in moles/liter) times time (in seconds). The term has been introduced for convenience and to

TABLE 2. *% Hybridization at saturation by cRNA prepared from chromatin prepared at different times after partial hepatectomy*

Hr after operation	% of DNA hybridized at saturation by cRNA
0	2.2
1.5	2.5
4	5.4
10	3.4
48	4.6

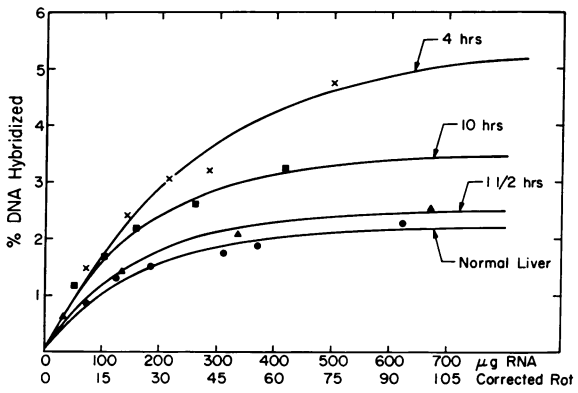

FIG. 3. Hybridization-saturation curves for cRNA from normal and regenerating rat livers.

avoid confusion with Cot, which cannot be properly used for RNA–DNA reactions.

RESULTS

Template activity

The ability of isolated chromatin (as compared to deproteinized DNA) to act as a template for *E. coli* RNA polymerase has been used for several years as a measure of the amount of the genome that is active *in vivo* (6). It has been well established that the genes transcribed *in vitro* are the same as those transcribed in the living tissue (7, 16–18) and, although the data are fragmentary, several attempts have been made to hybridize the RNA made *in vitro* to saturation. The values obtained in these experiments support the notion that the ability of chromatin to act as a template for *E. coli* RNA polymerase (template activity) is strongly correlated with the proportion of genes that are derepressed (Table 1).

We have measured the template activity of chromatin from various stages of regenerating rat liver, and a sample of the data is presented in Fig. 1. The maximum reaction velocity, V_m, at each time after hepatectomy was calculated from a double-reciprocal plot of each set of data, as shown in the inset. In Fig. 2, the V_m values from various stages of regenerating liver are compared with the V_m for normal liver. Each point represents the values obtained from 4–5 independent template assays, each one using the chromatin from 3–5 regenerating rat livers. The error bars represent the standard deviation of the data. There is an increase of about 35% in the template activity of chromatin of regenerating rat liver in the first 10 hr after hepatectomy. It appears, therefore, that a large number of previously repressed genes have been activated in this time interval. Presumably, these genes are those needed to prepare the cells for DNA synthesis and subsequent cell division, which begins 18 hr after the operation.

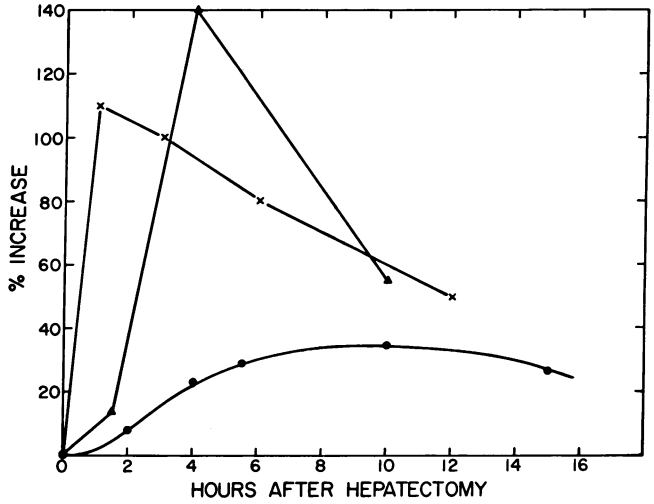

FIG. 4. ●——● Increase in template activity of regenerating rat-liver chromatin from Fig. 2. ▲-▲, % Increase in the hybridization-saturation values of cRNA from regenerating rat livers from Fig. 3. ×—×, % Increase in the hybridization-saturation values of rapidly-labeled nuclear RNA from Church and McCarthy (19).

cRNA hybridization data

Hybridization-saturation curves for cRNA isolated from regenerating rat livers at various times after hepatectomy are shown in Fig. 3. When RNA is in great excess, as in this case, the hybridization reaction is expected to follow first-order reaction kinetics. The curves drawn through the data were produced by a simple computer program that fits a first-order saturation curve to each set of experimental data. Table 2 gives the hybridization-saturation values calculated from this fit. The validity of this procedure rests on the assumption that the RNA sequences are all present in about the same concentration. Since this is undoubtedly not strictly true, these saturation values should be taken as minimum values. Rare RNA sequences will hybridize to saturation only at higher Rot values. It is clear, however, that the amount of information present in cRNA increase dramatically during the regeneration processes. A similar result was found by Church and McCarthy (19) for rapidly-labeled nuclear RNA, which we now believe to be the precursor of chromosomal RNA (5).

In Fig. 4, the three sets of data are combined. The early sequence of events in the regeneration process are clear from

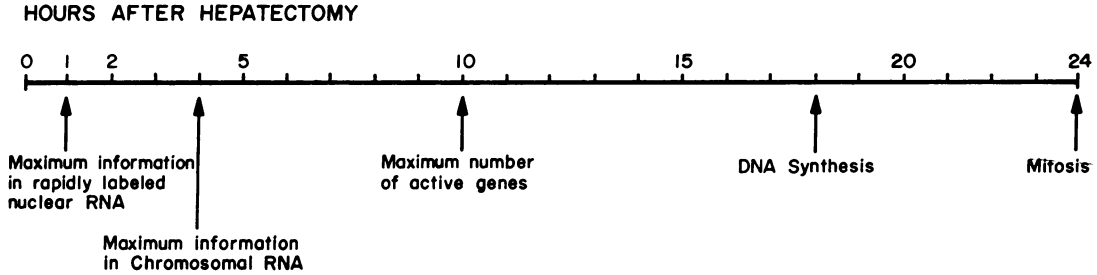

Fig. 5. Sequence of major nuclear events in the first 24 hr after partial hepatectomy.

the data of Fig. 4. The first new event (that we recognize so far) is the production of many new sequences of rapidly-labeled nuclear RNA. This rapidly-labeled RNA quickly breaks down and is followed by the appearance of a large number of new sequences in cRNA molecules. These are followed, in turn, by the activation of many new genes.

A representation of some of the major events in the first 24 hr of liver regeneration is shown in Fig. 5.

DISCUSSION

The regenerating liver is nearly ideal for the study of gene control. Regeneration is initiated by a well-defined stimulus, and many new genes are reproducibly activated within a few hours after hepatectomy. Since earlier evidence suggested that cRNA is involved in gene activation (7, 3), our first objective was to see if new cRNA sequences were produced in regenerating liver. The data of Fig. 3 show clearly that a large increase in such sequences does occur. This does not, of course, prove our hypothesis, but it does support our view that cRNA may be a gene activator.

One of the most striking aspects of the data presented here is that the sequence of events is exactly that predicted by the Britten-Davidson model of gene control (20). The central element of this model is a class of small RNA molecules responsible for activating genes. According to the model, in response to an outside stimulus, long tapes of activator RNA molecules are produced and specifically cleaved into functional activator RNA molecules, which then turn on the appropriate genes. Chromosomal RNA and rapidly-labeled nuclear RNA fit the requirements of activator RNA and its precursor. It is known that high-molecular-weight rapidly-labeled nuclear RNA and cRNA have a large fraction of their sequences in common (5), a finding that is difficult to explain unless rapidly-labeled nuclear RNA is the precursor of cRNA. Finally, the model requires that activator RNA hybridize to the repetitive fraction of the genome. Both RNAs are known to hybridize to the portion of the genome that contains repetitive DNA sequences. Thus, both RNAs hybridize to saturation at low Rot (RNA concentration $\times$ time) values characteristic of repetitive-sequence hybridization.

In conclusion, it is clear that the data presented here do not prove that the Britten-Davidson model is correct. There is much work to be done. However, our data do suggest that their model is an attractive one.

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