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Tissue Differences in Rat Chromosomal RNA

(gene activator/chromosomal RNA/chromatin/gene regulation)

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ABSTRACT Chromosomal RNA was isolated from several rat tissues by a new technique. It is shown by RNA–DNA hybridization that each tissue contains a distinct population of chromosomal RNA sequences. This finding is compatible with the proposal that chromosomal RNA is involved in the process of gene activation.

Chromosomal RNA (cRNA) is a class of nuclear RNA distinguished by its sequence diversity, small size, and high dihydropyrimidine content (1). Several experiments suggest that this RNA, apparently found in all eukaryotes, provides the specificity of gene control (2, 3). Assuming this to be true, there can be only two possibilities; cRNA is either a gene activator or a gene repressor.

In a typical eukaryotic cell, 90% or more of the genes are repressed. Thus, when one cell type is compared with another, the active genes are usually different, but the repressed genes are largely shared. Any gene regulator must reflect the genes it controls. Thus, it follows that if cRNA is an activator, every tissue should contain a distinctive set of cRNA sequences. If, on the other hand, it is a repressor, the cRNA sequences should be much the same from tissue to tissue. The hybridization data presented in this paper clearly show that the cRNA sequences of the rat tissues investigated are quite different. This result is consistent with the hypothesis that cRNA is a gene activator.

MATERIALS AND METHODS

Tissues. All tissues were obtained from 200–400 g, male, albino rats of the Sprague-Dawley strain purchased from Berkeley Pacific Laboratories. Regenerating livers were produced by surgical removal of the two largest lobes of the rats' livers by the method of Higgins and Anderson (4). Novikoff ascites-tumor cells were obtained from rats injected 6 days earlier with 0.5 ml of ascites fluid. The tumor line was maintained by serial transfer of 0.5 ml of ascites fluid on a 6-day cycle.

Chromatin. Crude chromatin was isolated from the solid tissues, as described by Marushige and Bonner (5), with three final washes in 0.01 M Tris·HCl buffer (pH 8). Nuclei were made from the ascites tumor cells with 0.5% Triton X-100 in saline–EDTA buffer (0.75 M NaCl–0.024 M EDTA, pH 8), and chromatin was isolated from the nuclei by three cycles of homogenization in a glass–Teflon homogenizer, followed by centrifugation at 10,000 rpm for 10 min, all in 0.01 M Tris·HCl buffer (pH 8).

Preparation of Chromosomal RNA (cRNA). cRNA was prepared from crude chromatin in two ways. The first, termed the "CsCl method", was exactly as described by Dahmus and McConnell (6). Chromatin was made 4 M in CsCl by the addition of 6 M CsCl–0.1 M Tris·HCl (pH 8), and centrifuged for 12–20 hr at 36,000 rpm in the Spinco 40 rotor. Protein floats to the top to form a pellicle or "skin", and cRNA is obtained from this skin by Pronase digestion, phenol extraction, and elution from DEAE-Sephadex A-25 with a 0.2–1 M NaCl gradient, all in 7 M urea. cRNA elutes as a sharp, symmetrical peak at 0.55 M NaCl. This method works very well for ascites tumor, but poorly for rat liver, and not at all for rat kidney. A second method was, therefore, developed.

This method, which will be referred to as the "DOC method", yields cRNA reliably from all tissues investigated. An equal volume of 1.7 M sucrose (enzyme grade, Mann Research) is added to the crude chromatin and the mixture is homogenized in a glass–Teflon homogenizer (3–5 strokes). Then, with constant stirring, 0.3 M sodium deoxycholate, is added dropwise, to a final concentration of 15 mM, and the mixture is stirred for an additional 30 min at 4°C. It is next centrifuged at 36,000 rpm for 6–12 hr in the Spinco 40 rotor. The supernatant is decanted and made 7 M in urea and 0.2 M in NaCl by the addition of solid urea and NaCl. DEAE-Sephadex, preequilibrated with 7 M urea–0.2 M NaCl, is then stirred in and a 0.9 × 20 cm column (10–50 g of tissue) is poured with the slurry. When the column has been poured and all the liquid has passed through, the cRNA is eluted with a 0.2-1 M NaCl gradient in 7 M urea. As with the CsCl method, the cRNA comes off in a homogeneous peak at 0.55 M NaCl. The RNA is routinely eluted a second time from DEAE-Sephadex to remove residual impurities. All cRNA prepared by this method that was 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.

In Vitro Labeling of cRNA. cRNA 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 0.2 ml of sodium phosphate buffer (pH 7.6), made with glass-distilled water (7). Incubation was performed for 6–12 hr at

Abbreviations: DOC, deoxycholate; SSC, standard saline citrate (0.15 M NaCl–0.015 M Na citrate).

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TABLE 1. *Protein content of DOC-extracted cRNA from several tissues*

Tissue	Protein, (%)
Calf thymus	7.3
Rat liver	5.0
Rat kidney	8.2
Ascites tumor*	1.0

* Treated with Pronase.

room temperature. 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 (8). 10 μg of ^{14}C-labeled DNA from the rat was applied to each filter in 6 × SSC (0.90 M NaCl–0.090 M sodium citrate). Retention was normally about 70%.

Hybridizations were done at 37°C in 50% formamide and 5 × SSC (0.75 M NaCl–0.075 M Na citrate); this temperature is about 22°C below the melting temperature of native rat DNA in this solution (9). Each reaction was performed in 0.2 ml of RNA solution with 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 (0.30 M NaCl–0.030 M Na citrate), digested with RNase (boiled pancreatic-ribonuclease A), washed again in 2 × SSC, dried, and counted in a Beckman liquid-scintillation spectrometer.

Chemical Analysis. Protein analysis was done by the method of Lowry *et al.* (10), with bovine-serum albumin as a standard. Ureido groups were analyzed by the method of Ceriotti and Spandrio (11) on RNA freed of all traces of urea by passage through Sephadex G-25.

Determination of the Half-Life of cRNA In Vivo. The method of Quincy and Wilson (12) was followed. Rats that had been infected with ascites-tumor cells for 5 days were injected intraperitoneally with 25 or 50 μCi of [^3H]orotic acid (17 Ci/mmol); after 2 hr, each rat was injected with 10 mg of unlabeled orotic acid. Ascites fluid was harvested at the desired time after labeling, cRNA was made from the ascites cells, and the specific activity of the cRNA was determined. A plot of the log of the specific activity versus time gave the apparent half-life of the RNA *in vivo*.

TABLE 2. *Yields of cRNA (in % of DNA) extracted from various chromatins by two different methods*

Tissue	CsCl	DOC	CsCl after DOC	Total cRNA present
Ascites	4	2.5	2.5	5
Liver	0.1	2	0.1	2
4-hr Regenerating liver	2	2	0.1	2
10-hr Regenerating liver	3	1.5	0.8	3
Kidney	0	0.8	0	0.8

TABLE 3. *Amounts of cRNA (in % of DNA) extracted from ascites chromatin by different concentrations of DOC*

	DOC concentrations, (%)				
	0.01	0.015	0.02	0.03	0.05
DOC supernatant	3	2.5	1.7	1.8	Chromatin destroyed
CsCl-extracted DOC pellet	2.3	2.5	2.6	3.2	DNA does not pellet under these conditions

Rot. The term Rot has been introduced for RNA–DNA hybridization reactions in which 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 (13). Rot stands for the concentration of RNA nucleotides in mol/liter times the time in seconds. The term has been introduced for convenience, and to avoid confusion with Cot, which cannot properly be used for RNA–DNA reactions.

RESULTS

Properties of cRNA isolated with DOC

Chromosomal RNA, isolated by either the DOC or the CsCl method, is characterized by its relatively small and uniform size as determined by chromatography on DEAE-Sephadex. Both methods give an RNA that elutes from DEAE-Sephadex at 0.55 NaCl in 7 M urea, and that contains about 8% of ureido-positive material by the colorometric test of Ceriotti and Spandrio (11). Several studies show that cRNA is sometimes bound covalently to protein (14, 15). In contrast to these findings, DOC-extracted cRNA, twice purified on DEAE-Sephadex, contains only a small amount of protein (Table 1);

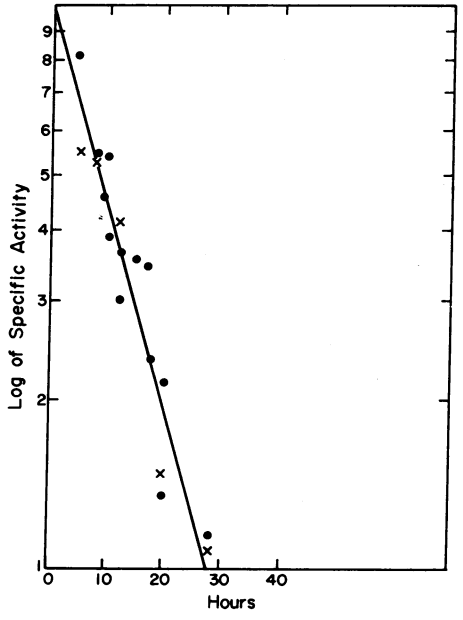

FIG. 1. Comparison of the decay kinetics of ascites cRNA *in vivo* isolated by both the CsCl method (●) and the DOC method (×). It is apparent that both methods give RNA that decays as a single component, with an apparent half-life of 8.5 hr. In these experiments, the ascites cells had a generation time of 17 hr, so that the real half-life of asictes cRNA (corrected for a doubling of mass every 17 hr) is also 17 hr.

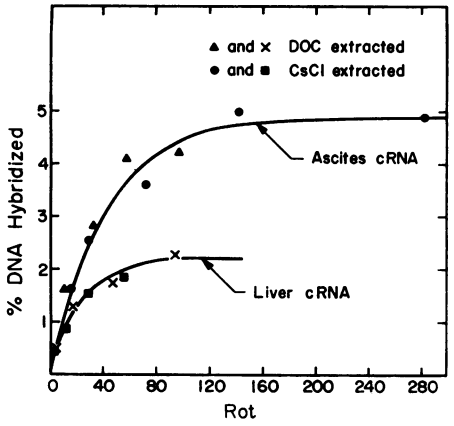

FIG. 2. Hybridization-saturation data that compare DOC-extracted and CsCl-extracted cRNA from liver and ascites tumor.

it is not known whether or not this protein is covalently bound to the RNA. It is possible that cRNA exists in the cell in both the bound and unbound states, and that only the protein-bound cRNA is isolated by the CsCl method. This hypothesis is borne out to some extent by the data of Table 2, which lists the yield of cRNA isolated by the two methods. 15 mM is such a low concentration of detergent that the chromatin is still intact in the pellet after extraction with DOC (16). The pellet may, therefore, be further extracted by the CsCl method. The results of this second cRNA isolation are given in the third column of Table 2. We were concerned that the large amount of cRNA from ascites tumor that is not extractable with 15 mM DOC might be simply an artifact of this particular concentration of detergent, but Table 3 shows that there appear to be two classes of cRNA present over a fairly wide range of DOC concentration. Although the proportions of the two classes are variable with different preparations, on the whole there appear to be about equal proportions of cRNA that are and are not extractable from ascites chromatin by low concentrations of DOC. The situation is much the same for regenerating liver, except that the proportion of the two classes changes with time (Table 2). If cRNA does exist in two states, however, they are part of a common pool. This is shown quite clearly by the data of Fig. 1, in which the logarithm of the specific activity of cRNA isolated at various times from rats that had received injections of [^{3}H]orotic acid is plotted against time. It is clearly seen that the decay rate of DOC-extracted cRNA *in vivo* is indistinguishable from that of CsCl-isolated cRNA.

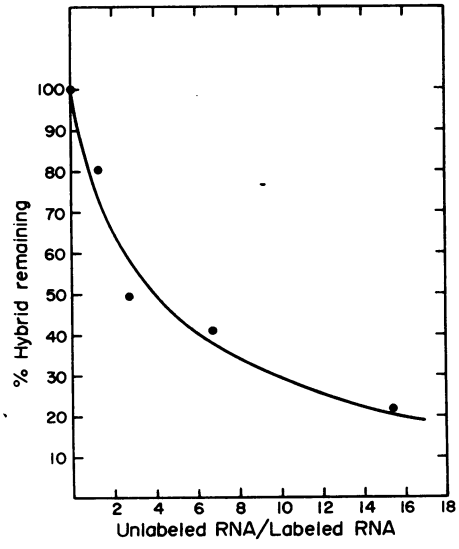

FIG. 3. Hybridization competition between CsCl-extracted [^{3}H]cRNA from liver (Rot = 55) and different amounts of unlabeled, DOC-extracted liver cRNA.

TABLE 4. *% of DNA hybridized at saturation by cRNAs of various tissues*

Tissue	RNA/DNA (%)
4-hr Regenerating liver	5.4
Ascites tumor	4.9
Liver	2.2
Kidney	2.2

The proof that cRNA isolated by the DOC method contains the same sequences as cRNA isolated by the CsCl method rests on the finding that their RNA–DNA hybridization kinetics are identical. Fig. 2 shows that the hybridization-saturation values are not affected by the method of isolation of either rat liver or ascites-tumor cRNA. Fig. 3 shows that DOC-isolated cRNA from rat liver competes well with CsCl-isolated cRNA from the same tissue.

Hybridization data for cRNA isolated from various tissues of the rat

Fig. 4 shows RNA–DNA hybridization saturation data from four rat tissues. When RNA is in great excess, this reaction is expected to follow approximate first-order reaction kinetics. The lines are produced by a simple computer program that fits a first-order saturation curve to each set of experimental data. The hybridization saturation values calculated from this fit are given in Table 4. The validity of this procedure rests on the assumption that the RNA sequences are all present in about the same concentration. This is almost certainly not true. The saturation values should thus be taken as minimum values, since rare RNA sequences will hybridize only at higher Rot

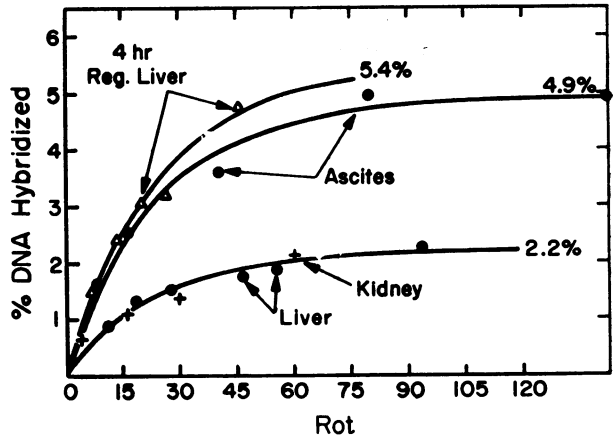

FIG. 4. Hybridization-saturation curves for rat-liver cRNA (●), rat-kidney cRNA (+), rat Novikoff ascites-tumor cRNA (●), and 4-hr regenerating rat-liver cRNA (△). The curves are first-order reaction kinetics fit by a computer to the data.

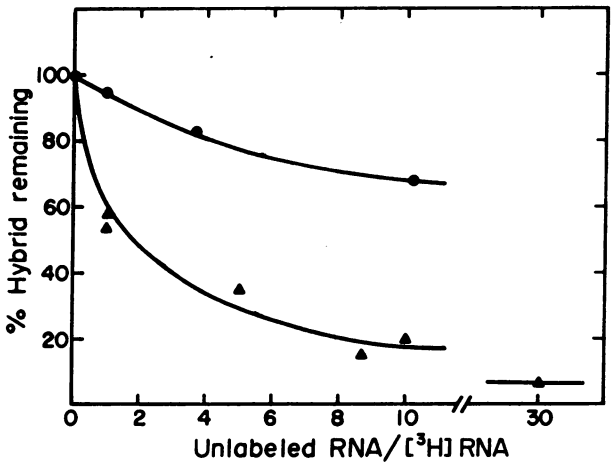

FIG. 5. Hybridization-competition comparison of cRNA from different tissues. (▲) labeled liver cRNA (Rot = 20) competed by unlabeled ascites-tumor cRNA. (●) labeled kidney cRNA (Rot = 30) competed by unlabeled liver cRNA.

values. However, unless more than half of the cRNA sequences present in liver and kidney are present in exceedingly low concentrations, it is clear that ascites and regenerating liver contain cRNA sequences not present in liver or kidney.

Fig. 5 shows, in addition, that about half of the sequences of kidney cRNA are absent or are present in very low concentration in rat liver, whereas nearly all of the liver sequences are included in the cRNA isolated from ascites tumor. Perhaps this last result is not too surprising, since Novikoff ascites tumor was originally a hepatoma.

Thus, we conclude from hybridization competition that kidney contains cRNA sequences not present in liver, and from hybridization saturation that ascites tumor and regenerating-liver cells contain cRNA sequences not present in normal liver or kidney cells. These results are consistent with those of Bonner and Widholm (17), which show that cRNA from pea buds differ from cRNA from pea cotyledons.

DISCUSSION

Several experiments have shown that cRNA is necessary for gene-specific reconstitution of isolated chromatin (2, 3). It is a short step to suppose that this cRNA is also responsible for gene regulation *in vivo*. Since in a typical mammalian tissue 90% or more of the genes are repressed, and since all higher eukaryotic cells contain histones that are almost certainly nonspecific repressors, it is probable that for the sake of efficiency, cells specifically activate those genes that are needed rather than to specifically repress those that are not. Any class of RNA responsible for gene activation must be composed of a distinctive set of sequences for each tissue type that correspond to the distinctive set of proteins that give a tissue its characteristics. In contrast, an RNA class responsible for gene repression should appear quite similar by the hybridization criterion when various cell types are compared, since nearly all of the repressed genes are contained in common by any two tissues.

Together with the known facts that cRNA is complementary to the middle repetitive-DNA sequences (18) and has a convenient length of about 50 bases (1), these findings are consistent with the hypothesis that cRNA provides one mechanism for introducing specificity of gene activation in higher creatures.

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