QUALITATIVE DIFFERENCES IN CHROMATINS OF ADULT TISSUES DEMONSTRATED BY ACTION OF DNase*

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SUMMARY

Treatment of chromatin from rat or mouse tissues with DNase rendered it tissue-specific in subsequent hybridization with H^3-labeled RNA's. It hybridized least with RNA from the same organ and most efficiently with RNA's from completely different organs. DNase-residual DNA's of forebrain were sufficiently specific to distinguish in this way between RNA's from different parts of the brain.

The technique of annealing or hybridizing DNA with RNA has been the basis for important studies designed to show progressive differentiation or other types of changes in transcription in eukaryotic cells and tissues (1, 2). However, the technique has certain limitations whether used as a simple saturation of fixed amounts of DNA with variable amounts of RNA, or as competition of two types of RNA for a limited number of annealing sites on DNA. The current techniques utilize denatured whole DNA preparations generally immobilized on discs of nitrocellulose for reaction with RNA or other DNA. A recently proposed alternative utilizing fragmented in vivo labeled DNA (3) is difficult to apply to study of transcriptive differentiation because of its complexity.

In searching for a practical technique that is more sensitive than those now available, we have developed the use of DNA prepared from chromatin that at one point is treated with DNase. On theoretical grounds it could be expected that the least DNA:RNA binding would occur between a DNase-resistant fraction of DNA and the RNA extractable from the same

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tissue. This would follow since, presumably, the transcriptively active regions of the chromatin (therefore the complementary RNA-binding sequences) would have been destroyed by hydrolytic action of the DNase. The interpretation that the DNase-susceptible areas in chromatin are those not protected by nucleoprotein and active in transcription was offered by Clark and Felsenfeld (4) recently. We feel that the experiments reported here confirm this interpretation and provide, in addition, evidence that DNase treatment affects qualitatively different areas of DNA in the chromatin of different adult tissues.

METHODS:

The basic design of the procedures described below involves the annealing or hybridization of H^3-uridine labeled RNA preparations from several different organs with DNase-fragmented DNA preparations, also from several organs. Graded quantities of RNA were used to the point of saturation of the fixed quantities of DNA. The DNA was prepared from chromatin that was treated with DNase prior to denaturation and immobilization on discs of nitrocellulose. The methods used for isolation of DNA and RNA are essentially as published by others, as indicated below, but with slight specified modifications.

For the labeled mouse RNA's one month-old males were each given 1 mCi of H^3-uridine (0.1 ml, > 25 Ci/mM) subcutaneously, 2 hours prior to death. Rapidly dissected liver and kidney were immediately frozen on dry ice and stored at $-80^{\circ}C$ until used. Similarly, 2 day-old female rats were given 1 mCi of H^3-uridine (0.01 ml, > 25 Ci/mM) by injection into the subdural space through the foramen magnum two hours prior to death. Brain and liver were rapidly dissected and frozen on dry ice.

The H^3-labeled RNA's from each organ were extracted by use of the technique of Scherrer and Darnell (5). Each RNA extract was treated twice, successively, with DNase and with pronase and passed through a Sephadex G-50 column.

The method of Marushige and Bonner (6) was used for isolation of chromatin from the adult female Sprague-Dawley rat and male Webster Swiss mouse tissues, as well as from Pseudomonas. Some of the chromatin was then treated with DNase I (5 µg/ml at 37°C, 1.5 hrs.) using the conditions described by Clark and Felsenfeld (4). The digestion was carried out in 5 mM sodium phosphate buffer, pH 6.7, containing 1 mM $MgCl_2$ at 37°C for 1 hr. Extraction of DNA from the various chromatin preparations (whether or not exposed to DNase) was by a procedure modified slightly from that described by Church and McCarthy (2). The DNase digestion was terminated by adding to the 5 mM phosphate buffer suspension of chromatin an amount of sodium dodecyl sulfate to produce a concentration of 1%, and sodium perchlorate to a concentration of 1 mM. To this viscous mixture an equal volume of chloroform-octanol (24:1) was added and shaken vigorously. It was centrifuged and the viscous aqueous phase collected. This procedure was repeated once. After addition of two volumes of ethanol to the aqueous phase as specified by Church and McCarthy, DNA from DNase-treated chromatin apparently was too fragmented to be spooled on a glass rod (2). Accordingly, we collected the DNA by centrifugation after the ethanol step.

The purified DNA was dissolved in 0.1 x SSC* to a concentration of 200 µg/ml and treated with 10 µg/ml boiled RNase for 1 hr. at 37°C, followed by treatment with preincubated pronase (10 µg/ml, 1 hr. at 37°C). This digestion was followed by addition of an equal volume of chloroform-octanol (24:1) shaking and centrifugation (3000 r.p.m., 10 min.). A second digestion with RNase and pronase followed repetition of the alcohol precipitation and solubilization in SSC. To the centrifuged supernatant, after the second digestion and treatment with chloroform-octanol, 2 volumes of cold ethanol was added to the aqueous layer, followed by centrifugation. The DNA suspension was denatured by heating at 95°C for 10 minutes and then rapidly cooled by dry ice — acetone immersion of the tube. The denatured DNA

*SSC, saline-citrate buffer (9) is 0.15 M NaCl, 0.015 sodium citrate, pH 7.2

suspension was diluted to a concentration of 10 µg/ml in 4 x SSC and filtered through and fixed upon nitrocellulose filters as described by Church and McCarthy (2).

The dried nitrocellulose filters were punched out into round discs 7 mm in diameter and samples analyzed spectrophotometrically for their DNA content by the diphenylamine reaction. Hybridization of the uniform discs containing immobilized whole, or DNase-fragmented DNA (about 20 µg per disc) with varying quantities of H^3-RNA was conducted in vials of 10 x 40 mm dimension in a volume of 0.2 ml of 4 x SSC, at 67°C for 17 hours (2). Fixation of the H^3-RNA on the washed DNA discs was measured by counting in a Beckmann LS-233 liquid scintillation counting system.

RESULTS AND DISCUSSION

When whole denatured DNA's, prepared from three different rat tissues are used (forebrain, hindbrain, liver), these three kinds of DNA bind a particular (forebrain) RNA to an equal degree (Fig. 1A). In contrast, DNA derived from DNase-treated chromatin not only binds less RNA, but also it binds it differentially and selectively, reflecting the tissue sources of the DNA and the RNA (Fig. 1B and 2). That is, when rat forebrain H^3-RNA was incubated with DNase-pretreated DNA's from forebrain, hindbrain and liver, it bound least with its homologous DNA, best with liver DNA and to an intermediate degree with DNA from another part of the brain (Fig. 1B). Further, in an experiment with mouse tissues, it was found that hepatic DNA, prepared from DNase treated chromatin, bound kidney H^3-RNA somewhat more efficiently than its homologous liver H^3-RNA (Fig. 2).

In both the rat experiment, and in the mouse experiment, DNase-pretreated DNA still bound about 13% of the homologous RNA that whole DNA will bind. Theoretically, zero binding might be expected. It is possible that this level of binding is due to incomplete action of the DNase. It is also possible that there are some inactive or repressed DNA sequences that reiterate certain of the sequences being actively transcribed. These would

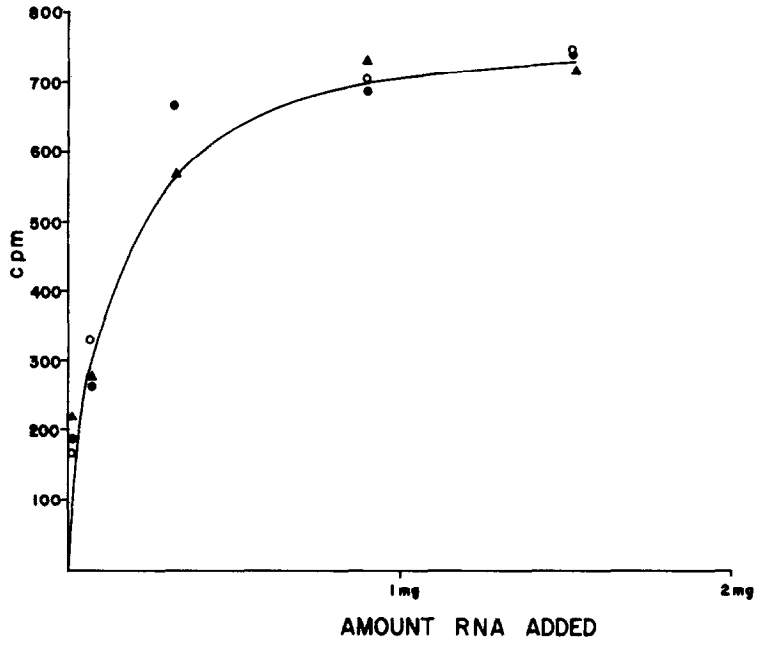

Fig. 1A

Hybridization to saturation between labeled rat forebrain H^3-RNA and DNA's prepared from chromatins of rat forebrain, hindbrain and liver without prior DNase treatment. Values are expressed in cpm corrected for the amount of DNA per filter disc.

—●— Labeled RNA from forebrain, DNA from forebrain chromatin (15.5γ/disc)

—○— Labeled RNA from forebrain, DNA from hindbrain chromatin (20.4γ/disc)

—▲— Labeled RNA from forebrain, DNA from liver 14.0γ/disc)

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be protected from DNase action on chromatin, but would become available for subsequent binding with RNA after the procedures employed here. The 13% residual DNA:RNA binding is well above the non-specific or "noise-level" binding (2 to 3%) seen by exposing the same RNA's to whole bacterial DNA prepared from Pseudomonas (Fig. 1B,2).

It is of possible significance that maximum hybridization of such residual DNA's with RNA's from completely different organs was also relatively uniform, 27.5% or 29.8% of the value when whole DNA was used (see Fig. 1B and 2). This was true when rat liver residual DNA was annealed with rat

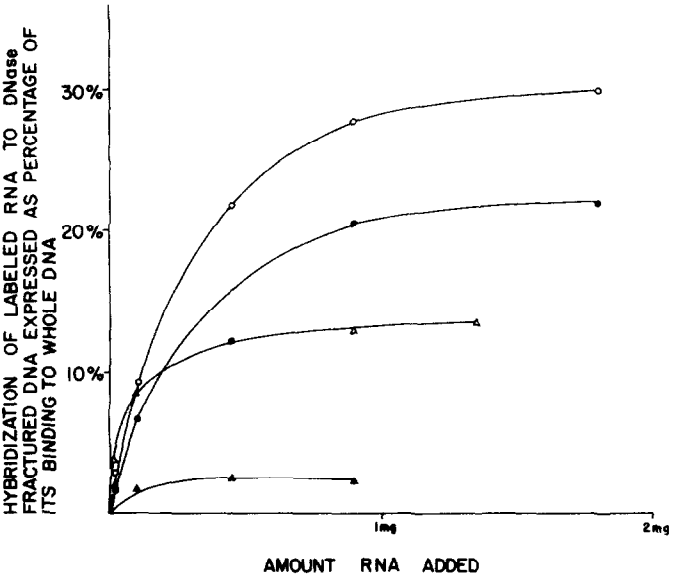

Fig. 1B

Hybridization to saturation of H^3-labeled rat forebrain RNA and DNA's isolated from chromatins of rat forebrain, hindbrain, and liver after DNase treatment. Values are expressed as the fraction (percent) of the hybridization between the same amounts of H^3-labeled rat forebrain RNA and DNA's isolated from intact chromatin of rat forebrain, hindbrain or liver.

—o—o— H^3-RNA of forebrain, DNA from liver (16.2γ/disc)

—●—● H^3-RNA of forebrain, DNA from hindbrain (13.0γ/disc)

—△—△— H^3-RNA of forebrain, DNA from forebrain (13.3γ/disc)

—▲—▲— H^3-RNA of forebrain, DNA from Pseudomonas (12.8γ/disc)

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forebrain RNA, as well as when mouse liver residual DNA was paired with mouse kidney RNA. It is of interest that residual rat hindbrain DNA bound rat forebrain RNA maximally at an intermediate 21.9% level. If these figures may be taken as expression of degrees of qualitative relatedness between transcription in different organs, they appear to indicate what one might expect: There is some degree of qualitative overlap between transcription in two different organs. In two different regions of one organ, forebrain and hindbrain, this similarity is greater than in comparing transcriptive activity in two completely different organs.

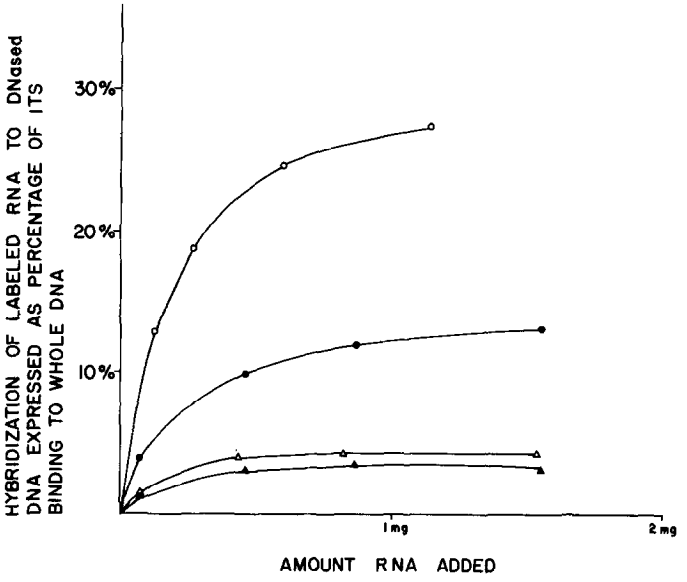

Fig. 2

Hybridization to saturation of H^3-labeled mouse liver and kidney RNA, with fixed quantities of DNA isolated from chromatin of mice liver after DNase treatment. Values are expressed as the fraction (percent) of the hybridization between the same amounts of labeled mouse liver or kidney RNA and mouse liver DNA isolated from intact (not treated with DNase) chromatin.

—o—o— H^3-RNA of kidney, DNA of liver (13.5γ/disc)

—●—● H^3-RNA of liver, DNA of liver (13.5γ/disc)

—△—△— H^3-RNA of kidney, DNA from Pseudomonas (12.8γ/disc)

—▲—▲— H^3-RNA of liver, DNA from Pseudomonas (12.8γ/disc)

The fact that transcriptive patterns in forebrain may be differentiated from those of hindbrain (Fig. 1B) opens up many possibilities for further study of brain function that have been approached with difficulty until now. For example, it has been found (7) that treatment of newborn female rats with male hormone causes permanent changes in brain function that are interpreted as an induced abnormal differentiation. On the basis of "classic" DNA:RNA hybridization experiments (8) it has remained unclear whether the hormone treatment causes a permanent change in brain transcription.

It would appear that by use of DNase-pretreated DNA a much more sensitive

tool is now available for determination of qualitative changes in patterns of regional transcriptive activity in the brain in different states of development or function.

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