The Hybridization Capacity of Ribonucleic Acid Produced during Hormone Action

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1. Measurements of hybridization with homologous DNA were used to assess the nature of the RNA synthesized during hormone action in several systems. 2. When increasing amounts of pulse-labelled rat liver nuclear RNA were annealed with constant amounts of DNA, saturation was not achieved even with RNA/DNA ratios of up to 180:1, which is taken to indicate great diversity in the species of labelled RNA molecules. In the converse experiment, when the DNA/RNA ratio was varied up to 20:1, a plateau of hybridization was observed, and the non-hybridizing RNA is believed to represent chiefly ribosomal and ribosomal precursor species. 3. In the livers of hypophysectomized and thyroidectomized rats treated with growth hormone and tri-iodothyronine, and in whole *Xenopus* larvae during induced metamorphosis, the synthesis of non-hybridizing RNA was consistently stimulated more than that of hybridizing RNA. This is interpreted as reflecting preferential synthesis of ribosomal RNA in response to these hormones.

An early and essential action of a wide variety of anabolic or developmental hormones is the stimulation of synthesis of RNA in their target cells (Tata, 1966). Though the selective synthesis of m-RNA† may be a determinant of specificity in hormone-induced protein synthesis, it is clear that all classes of RNA are affected. Indeed, with some hormones there is evidence that the production of r-RNA is especially sensitive to stimulation (Liao & Lin, 1967; Tata, 1967a). Measurement of hybridization, or the capacity of radioactively labelled RNA to anneal with homologous DNA, is a sensitive means of RNA characterization that has been used to assess the extent of m-RNA synthesis during different stages of development and regeneration (Denis, 1966a,b; Church & McCarthy, 1967a,b), as well as during response to hormones (Drews & Brawerman, 1967a,b). We now report experiments using this technique that indicate preferential stimulation of r-RNA synthesis in three systems of hormone-induced growth and development.

METHODS

DNA. DNA was prepared from liver nuclei of rats and bullfrogs (*Rana catesbeiana*) by the method of Marmur (1961) and from erythrocytes of South African clawed toads

(*Xenopus laevis*) by the method of Birnstiel, Wallace, Sirlin & Fischberg (1966).

The DNA was treated at 37° for 1½ hr. with ribonuclease (100 µg./ml.) (bovine pancreatic, 2 × crystallized; Boehringer Corp. Ltd., London, W. 5; previously heated at 80° for 10 min.) and α-amylase (100 µg./ml.) (*Bacillus subtilis*, crystalline; Sigma Chemical Co., St Louis, Mo., U.S.A.), and then for a further 1½ hr. with Pronase (100 µg./ml.) (Calbiochem, Los Angeles, Calif., U.S.A.). It was then deproteinized by addition of 1% sodium dodecyl sulphate and shaking twice with water-saturated phenol, and precipitated with 0.1 M-NaCl and 2 vol. of ethanol. After the DNA was redissolved and reprecipitated, its contents of contaminating RNA and protein were generally less than 1%, and glycogen was less than 5%. For the hybridization experiments, DNA was denatured with 0.2 N-NaOH, neutralized, diluted to 1–5 µg./ml. in 5 × SSC and trapped by filtering through 25 mm.-diameter nitrocellulose membranes (type B-6; Carl Schleicher and Schuell Co., Keene, N.H., U.S.A.; or type MF-50; Sartorius-Membranfilter, Göttingen, Germany). The amount of DNA bound was determined by extracting it from sample filters from each batch with 0.5 N-HClO₄ at 70° and measuring the extinction at 260 mµ. The filters were dried by the procedure of Gillespie & Spiegelman (1965).

RNA. RNA was labelled *in vivo* with [³H]orotic acid (6.9 c/m-mole; The Radiochemical Centre, Amersham, Bucks.) for rats and with [³H]uridine (2.7 c/m-mole; The Radiochemical Centre) for tadpoles. Liver nuclear RNA was extracted from either 'crude nuclei' (sedimented once from 0.32 M-sucrose) or 'purified nuclei' (resuspended and sedimented from 2.1 M-sucrose) isolated by the method of Widnell & Tata (1964). Cytoplasmic RNA was extracted from two subfractions of microsomes, the smooth and rough membranes, which were prepared by the method of Tata & Williams-Ashman (1967). The nuclei or the microsomal subfractions were suspended in 0.05 M-sodium acetate buffer,

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† Abbreviations: m-RNA, messenger RNA; t-RNA, transfer RNA; r-RNA, ribosomal RNA; SSC, 0.15 M-NaCl–0.015 M-sodium citrate, adjusted to pH 7 (2 × SSC and 5 × SSC contain the solutes at twice and five times this concentration respectively).

pH 5.0, and, after addition of a few milligrams of bentonite and macaloid, were treated with 0.5% sodium dodecyl sulphate and 1 vol. of water-saturated phenol by mixing first at room temperature and then for several minutes while the temperature within the mixture was raised to 55°. *Xenopus* RNA was extracted from whole larvae, which were homogenized in 0.1 M-NaCl in 0.05 M-sodium acetate buffer, pH 5.0, containing sodium dodecyl sulphate (1.0%); further steps were the same as for liver nuclear RNA.

The suspensions were chilled and centrifuged and the aqueous layers drawn off. The phenol and interphase were re-extracted with acetate-sodium dodecyl sulphate at 55°, and the combined aqueous phases were then re-treated with fresh phenol. RNA was precipitated with ethanol, redissolved in a small volume of the acetate buffer, centrifuged at 55000g for 20 min. to remove glycogen and then precipitated overnight with 10% NaCl at -6° (Hadjivassiliou & Brawerman, 1965). This last step separates RNA of high molecular weight from t-RNA, DNA and further glycogen. In many experiments the RNA was used at this stage; if, however, analysis (Burton, 1956) showed more than 1% of DNA as a contaminant, the RNA was treated with deoxy-ribonuclease (20 µg./ml. at 37° for 1 hr.) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; electrophoretically purified), followed by Pronase (100 µg./ml. at 37° for 1 hr.), and was then deproteinized with sodium dodecyl sulphate and phenol and passed through a column (1 cm. × 70 cm.) of Sephadex G-50 to remove the digestion products.

Hybridization of RNA and DNA. This was carried out essentially by the method of Gillespie & Spiegelman (1965). Annealing took place in 1.5 ml. of 2 × SSC at 67° for 16 hr. Unhybridized RNA was removed with ribonuclease as described by Gillespie & Spiegelman (1965) except that the membranes were washed by gentle shaking with 2 × SSC instead of by filtration. When the ribonuclease was omitted and replaced by a wash with 2 × SSC at 67° (Church & McCarthy, 1967a) the bound RNA radioactivity was roughly doubled and the relative differences in extent of hybridization of different RNA preparations were retained, though the magnitude of differences seemed slightly decreased.

Fig. 1. Hybridization with constant DNA and increasing rat liver nuclear RNA. [³H]RNA (specific radioactivity 86 counts/min./µg.) was prepared from crude liver nuclei of normal rats killed 45 min. after the injection of 250 µC of [³H]orotic acid and was assayed for hybridization with filters carrying 2 µg. of DNA.

Blank filter membranes and membranes carrying heterologous (*Escherichia coli*) DNA were included in all experiments and generally gave less than 2% of the radioactivity of the homologous system. Bound RNA was measured by liquid-scintillation counting with an efficiency of 18% and a background of 8 counts/min.

Animals and treatment. Male Sprague-Dawley rats were from the stock bred at the National Institute for Medical Research. Treatment of hypophysectomized or thyroidectomized rats with growth hormone or tri-iodothyronine or both is described in the Figure legends and followed the same pattern as used in previous work on the effect of hormones on RNA and protein synthesis (Tata & Widnell, 1966; Widnell & Tata, 1966; Tata & Williams-Ashman, 1967). Bullfrog tadpoles were obtained from Connecticut Valley Biological Supply Co., Southampton, Mass., U.S.A., and metamorphosis was induced precociously with tri-iodothyronine, as described by Tata (1967b). *Xenopus* tadpoles were reared in the laboratory after artificial ovulation and fertilization induced in mature adults obtained from South African Snake Farm, Fishoek, Cape Province, South Africa.

RESULTS AND DISCUSSION

Quantitative relationships in hybridization. In the experiment shown in Fig. 1, we attempted to find a plateau in the capacity of rat liver RNA to hybridize with radioactive RNA from crude liver nuclei, but this was not achieved with RNA/DNA ratios up to 180:1. This behaviour presumably signifies that the labelled RNA contains a wide variety of molecular species many of which are present in very small proportions, so that DNA sites remain unsaturated within the range of ratios tested. This observation shows that, with heterogeneous animal RNA preparations, very high RNA/DNA ratios will be required to demonstrate effective competition between RNA preparations, or presaturation of DNA. The failure of Birnboim, Pène & Darnell (1967) to achieve presaturation with RNA/DNA ratios 12:1 or 14:1 is therefore understandable, and should not be taken as evidence against specificity in hybridization experiments with animal RNA.

In Fig. 2 are shown two representative experiments with rat liver nuclear RNA, in which a constant amount of radioactive RNA was assayed for duplex formation with increasing amounts of homologous DNA bound on filters. In such experiments, a definite plateau is achieved, at which the presentation of more DNA leads to little more RNA being bound (Fig. 2a). The experiment of Fig. 2(b) differs in that the RNA was prepared from nuclei freed of contaminating cytoplasm, so that diluting non-hybridizing RNA has been removed, and, as one would expect, higher DNA/RNA ratios are required. In this experiment, a flat plateau was not achieved, but in other experiments with RNA similarly prepared this was attained with DNA/RNA ratios less than 20:1.

Fig. 2. Effect of hormone treatment on hybridization capacity of rat liver nuclear RNA. Hypophysectomized rats (120–130g.) were injected with tri-iodothyronine (0.2 µg./g. 44–48 hr. and 12 hr. before being killed) and human growth hormone (0.6 µg./g. 22–24 hr. and 12 hr. before being killed). RNA was labelled by injection of [³H]orotic acid (250 µC/rat) 45 min. before killing. The ratio of DNA to RNA in the hybridization tests was plotted against the percentage of input RNA radioactivity hybridized. (a) RNA extracted from crude liver nuclei, 5 µg./filter: ○, hypophysectomized control (specific radioactivity of RNA 316 counts/min./µg.); □, hormone-treated (specific radioactivity of RNA 738 counts/min./µg.). (b) RNA extracted from purified liver nuclei, 1.5 µg./filter: ○, hypophysectomized control (specific radioactivity of RNA 2004 counts/min./µg.); □, hormone-treated (specific radioactivity of RNA 3630 counts/min./µg.).

In a number of such experiments with rat liver nuclear RNA, in which constant amounts of RNA were hybridized with various quantities of DNA, hybridization plateaus ranging from 2% to 6% of the input RNA radioactivity were observed. The labelled RNA can therefore be divided into fractions that hybridize and that do not hybridize, or do so only slightly, under the conditions used. A similar behaviour has been described in the hybridization of mouse liver nuclear RNA by Hoyer, McCarthy & Bolton (1963), and in the hybridization of RNA obtained from different developmental stages of the *Xenopus* embryo by Denis (1966a). Because of the high content of ribosomal species in the bulk RNA and the small fraction of their cistrons in the DNA, only negligible proportions of r-RNA and ribosomal

precursor RNA species would be expected to hybridize at these relatively low DNA/RNA ratios. The m-RNA species, on the other hand, not being present in such excess relative to their cistrons, will be expected to hybridize. We therefore interpret the observed plateau value as a measure of the non-ribosomal content of the high-molecular-weight nuclear RNA synthesized during the labelling period. There is, however, the possibility that the non-hybridizable RNA may be m-RNA or DNA-like RNA that is transcribed on non-reiterated genes present in extremely low concentrations. Waring & Britten (1966) have shown that much of mouse DNA consists of repeating nucleotide sequences. However, under conditions of hybridization similar to those we have employed, Denis (1966a) has found that the base composition of hybridizing RNA from *Xenopus* embryos is DNA-like, whereas that of non-hybridizing RNA is indicative of r-RNA and t-RNA. In our experiments, t-RNA was removed in the preparative procedure. The absolute value for the hybridization plateau is difficult to interpret, since it is sensitive to experimental conditions such as the volume in which annealing is carried out and whether ribonuclease is included in the washing treatments. The differences in extent of hybridization of different RNA preparations, however, are highly reproducible despite variations in technique, and must reflect real differences in the RNA.

Hormonal effects on rat liver RNA synthesis. Growth hormone and tri-iodothyronine are both known to stimulate markedly the synthesis of RNA in the livers of hormone-deficient (i.e. hypophysectomized or thyroidectomized) rats (Talwar, Gupta & Gros, 1964; Tata & Widnell, 1966). In these experiments we chose to use a combined hormonal treatment, since the two hormones administered together to hypophysectomized rats produce additive and maximal stimulations of both RNA and protein synthesis in the liver (Widnell & Tata, 1966; Tata & Williams-Ashman, 1967). Treatment of hypophysectomized rats with tri-iodothyronine and growth hormone stimulated the total incorporation of [³H]orotic acid into liver nuclear RNA (two- to four-fold increases in specific radioactivity) as well as the synthesis of RNA hybridizing under the conditions described (Figs. 2a and 2b: counts/min. in hybridized RNA). The percentage hybridization of the labelled RNA, however, was invariably decreased after hormone treatment. We believe this result indicates that the hormone treatment stimulated synthesis of r-RNA more than that of other kinds of RNA.

Two experiments with hydrocortisone (1 or 1.8 mg./100 g. injected into hypophysectomized rats 3 hr. before they were killed) gave similar results: the percentage hybridization of the labelled nuclear

Fig. 3. Effect of administration of tri-iodothyronine to rats on hybridization capacity of liver cytoplasmic RNA. Thyroidectomized rats (150–170 g.) were injected with tri-iodothyronine (0.2 $\mu\text{g./g.}$) at 66 and 38 hr. before being killed. Cytoplasmic RNA was labelled by the injection of 350 μC of [^3H]orotic acid per rat 11.0 hr. before the rats were killed. RNA was extracted separately from smooth microsomal membranes ($\circ$ and $\bullet$) and rough microsomal membranes ($\square$ and $\blacksquare$). Smooth-membrane RNA (7 $\mu\text{g./filter}$): $\circ$, from control thyroidectomized rats (specific radioactivity of RNA 450 counts/min./ $\mu\text{g.}$); $\bullet$, from tri-iodothyronine-treated rats (specific radioactivity of RNA 969 counts/min./ $\mu\text{g.}$). Rough-membrane RNA (16 $\mu\text{g./filter}$): $\square$, from control rats (specific radioactivity of RNA 220 counts/min./ $\mu\text{g.}$); $\blacksquare$, from hormone-treated rats (specific radioactivity of RNA 395 counts/min./ $\mu\text{g.}$). Co-ordinates are as indicated in Fig. 2.

RNA was somewhat decreased (to about 80% of that of controls), suggesting that the steroid also preferentially stimulates synthesis of r-RNA. Drews & Brawerman (1967*a,b*) have concluded from RNA fractionation studies that hydrocortisone enhanced the synthesis of r-RNA and m-RNA by about the same extent. Though they performed hybridization tests with competition in order to study specificity in m-RNA production, after treatment with both hydrocortisone and growth hormone, the hybridization efficiency of their RNA preparations was not reported.

In another group of experiments, we tested cytoplasmic RNA prepared from rat liver before and after hormone treatment. This was important because of recent evidence from hybridization studies indicating that in normal mammalian cells there is a high degree of restriction of nuclear RNA to the nucleus (Shearer & McCarthy, 1967; Drews, Brawerman & Morris, 1968). In the experiment illustrated in Fig. 3, we tested RNA isolated separately from the smooth and rough liver microsomal membranes of thyroidectomized rats with and without treatment with tri-iodothyronine. Again, it was not possible to show any enrichment

Table 1. Effects of induction of metamorphosis on hybridization of tadpole liver nuclear RNA

Tadpoles of *Rana catesbeiana* were given tri-iodothyronine (1 $\mu\text{g./animal}$ injected plus 10 $\mu\text{g./l.}$ in the water) and then at the times indicated were injected with 52 μC of [^3H]uridine and killed 3 hr. later. RNA was prepared from nuclei obtained from pooled livers of 10–15 tadpoles and was tested for hybridization with 6.6 $\mu\text{g.}$ of RNA/filter. Each value for the hybridization plateau is the mean from three tubes with 36 $\mu\text{g.}$ of DNA and three tubes with 47 $\mu\text{g.}$ of DNA.

Time after hormone (days)	Sp. radio-activity of RNA (counts/min./ $\mu\text{g.}$)	Hybridization plateau	
		(counts/min./ $\mu\text{g.}$ of RNA)	(% of input radioactivity)
0 (control)	30	2.1	6.9
2	70	3.8	5.4
6	155	4.9	3.2

in the readily hybridizable RNA after hormone treatment. Other experiments showed consistently that the hybridization capacity of total microsomal RNA (labelled for 8, 11 and 17 hr.) is about 20–30% of that exhibited by nuclear RNA (labelled for 45 min.), which may result from the failure of certain RNA species to pass out from the nucleus as well as the more rapid turnover of m-RNA than of r-RNA in the cytoplasm. The RNA from smooth membranes, lacking ribosomes, exhibits a higher hybridization efficiency at low DNA/RNA ratios than that from rough membranes. Several workers have described RNA associated with microsomal membranes (Bergeron-Bouvet & Moulé, 1966; Shapot & Pitot, 1966; Tata, 1967*c*) and, though the biological significance of this RNA is not yet clear, it is noteworthy that its rate of synthesis is very sensitive to hormone administration (Tata, 1967*b,c*).

RNA synthesis in hormone-induced amphibian metamorphosis. We applied the same test of hybridization to the RNA produced in nuclei of livers of bullfrog tadpoles (*Rana catesbeiana*) in which metamorphosis was induced precociously with tri-iodothyronine, which is known to bring about the synthesis of urea-cycle enzymes, serum albumin and other proteins in tadpole liver (Bennett & Frieden, 1962). It has been shown (Nakagawa, Kim & Cohen, 1967; Tata, 1967*b*) that maximal stimulation of the rate of synthesis of nuclear RNA occurred 2 days after introducing tri-iodothyronine, whereas new enzyme formation was maximal at 6 days. We therefore prepared labelled nuclear RNA at both 2 and 6 days of induced metamorphosis. The results in Table 1 are quite analogous to those from rat liver: at both times of hormone action the production of hybridizing RNA is elevated, but

Table 2. *Hybridization of total Xenopus larval RNA at two different stages of induced metamorphosis*

The 3-4-week-old tadpoles of *Xenopus laevis* were immersed in water containing tri-iodothyronine ($5\mu\text{g./l.}$) to induce metamorphosis. Control and treated tadpoles (50-80 larvae/batch) were used for preparation of RNA 20 hr. after the injection of $5\mu\text{C}$ of [^3H]uridine. Hybridization was performed with $5.2\mu\text{g.}$ of RNA/filter carrying $32\mu\text{g.}$ of *Xenopus* DNA. Each value for the hybridization plateau is the mean of three determinations performed in duplicate.

Time after hormone (days)	Sp. radio-activity of RNA (counts/min./ $\mu\text{g.}$)	Hybridization plateau	
		(counts/min./ $\mu\text{g.}$ of RNA)	(% of input radioactivity)
0 (control)	87	4.2	4.8
3	345	13.0	3.8
5	629	15.7	2.5

the production of non-hybridizing RNA is stimulated to an even greater extent.

Similar results were also observed when total RNA from whole *Xenopus* larvae was prepared before and after the induction of metamorphosis (Table 2). Since thyroid hormone induces the synthesis of different proteins in different tissues of the embryo (Bennett & Frieden, 1962), a wider variety of newly synthesized m-RNA molecules would be present in RNA extracted from the whole animal than from liver alone. The decrease in hybridization efficiency in whole *Xenopus* RNA suggests that the synthesis of r-RNA is also preferentially stimulated in tissues other than the liver.

Conclusions. These results support other lines of evidence in showing that the synthesis of different kinds of RNA is involved in responses to hormones (Tata, 1966). The stimulation of synthesis of readily hybridizable RNA suggests production of new or more m-RNA molecules as a result of hormone treatment. To what extent the additional hybridizable RNA serves as templates for protein synthesis, however, is uncertain in view of recent evidence on RNA of unknown function that does not escape from the nucleus (Shearer & McCarthy, 1967; Drews *et al.* 1968). In any case, the greater stimulation of synthesis of non-hybridizing (at low DNA/RNA ratios) RNA indicates a preferential effect of hormones on production of r-RNA and confirms conclusions drawn from other methods of characterization (Tata, 1966, 1967a; Liao & Lin, 1967). Increased production of ribosomes may be required for the transport of RNA from the nucleus to the cytoplasm as well as for an enhanced synthesis of hormone-specific proteins, whether or not new m-RNA species and new kinds of protein are involved.

We must qualify the generality of these results by pointing out, first, that it is quite possible that, in both mammalian and amphibian systems, a preferential stimulation of m-RNA synthesis may take place at very early times after hormone administration, followed by a massive synthesis of r-RNA giving the phenomenon that we have observed. If only a few species of m-RNA were involved in the early effect, it might not be detectable by this technique, especially if the hormone were preferentially to enhance the synthesis of m-RNA of non-reiterated genes present in extremely low concentrations. It is also possible that highly specialized tissues such as the eye of *Xenopus*, whose role in metamorphosis depends on the production of a few unique proteins, may synthesize proportionately more m-RNA in response to thyroid hormone. Further, the type of analysis reported here does not permit us to say anything about the qualitative distribution of m-RNA. The hybridizing RNA produced in response to the hormones probably includes m-RNA of a certain range of species, some of which may be new. Competition studies of the type described by Denis (1966b), Drews & Brawerman (1967a,b) and others are beginning to throw light on this important aspect of the problem.

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