Polyphasic Changes in Incorporation of Precursors into Ribonucleic Acid of Oestradiol-Stimulated Mammary Gland

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1. At 3 weeks after ovariectomy, mammary glands (5th pair) of adult Swiss mice show (i) no significant decrease in weight, (ii) 20% of the original rate of incorporation of [3 H]-uridine into RNA (after a 30 min pulse), and (iii) 90% of the original rate of incorporation of L-[3 H]leucine into protein (after a 15 min pulse). 2. A single injection of oestradiol-17 β into these ovariectomized mice produces, during the next 17 h, a series of discrete bursts of increased incorporation of [3 H]uridine into mammary-gland RNA; the bursts, which are variable in height, reach peaks at approx. 1, 9, 12 and 16 h after hormone administration; an increase is already detected at 15 min, the earliest time-point investigated; each burst lasts for approx. 2 h. There is no significant stimulation of [3 H]uridine incorporation into RNA of liver and quadriceps femoris muscle. 3. Nuclear incorporation of [3 H]UTP into RNA of mammary gland *in vitro* is linear with time for up to 20 min at 15°C; it requires CTP, GTP and ATP and is inhibited by actinomycin D. Also, the incorporation is strongly inhibited by α -amanitin in high salt concentrations but only weakly in low salt concentrations, a result indicating that RNA polymerase II activity predominates in high salt, whereas RNA polymerase I activity predominates in low salt concentrations. Injection of oestradiol-17 β *in vivo* followed by measurement of nuclear RNA synthesis *in vitro* shows a definite increase in both RNA polymerase activities 30 min after oestradiol-17 β injection, the earliest time-point investigated, a higher increase at 1 h, a decline at 4 h, and again a large increase at 12 h. These results in general agree with the changes in precursor incorporation into RNA measured directly in the animal and suggest that changes in [3 H]uridine uptake into RNA are not precursor-pool-dependent.

DNA synthesis and cell proliferation in the mammary gland of adult mice slow down and come to a virtual stop after ovariectomy; the cells go into a 'standby' or G₀ state, ready to re-enter the cell-replication cycle on administration of oestrogen to the animals (Bresciani, 1964, 1965). After oestradiol-17 β injection, DNA synthesis and mitosis in the gland resume in a synchronous fashion after a 48–72 h lag, with the number of responsive cells doubling in the next 72–96 h (Bresciani, 1971). Cell proliferation resulting in duct elongation appears to be the main response of the gland to oestrogen (Bresciani, 1968) and it is prevented when early RNA synthesis is stopped (Bresciani *et al.*, 1969).

Exceedingly little is known about the mechanism of the above effect of oestrogen on cell proliferation in the mammary gland. We know that the gland contains specific oestrogen-receptor proteins (Bresciani & Puca, 1965; Puca & Bresciani, 1969; Shyamala & Nandi, 1972) and that the oestrogen-receptor complexes, once formed in the cytoplasm, rapidly move to the nucleus (Puca *et al.*, 1967). However, there is a

gap in our knowledge of biochemical events after the initial interaction of the hormone with the gland and preceding the later surge of DNA synthesis and cell division.

In the present paper we report that injection of oestradiol-17 β into ovariectomized mice starts, within minutes, a complex response consisting of several discrete bursts of increased [3 H]uridine incorporation into total RNA. Incorporation of [3 H]UTP into RNA of isolated nuclei shows similar results. It is suggested that these subsequent discrete bursts represent increased RNA synthesis and reflect initiation and orderly expression of a programme inscribed into DNA, eventually leading to DNA replication and cell division.

Experimental

Materials

Oestradiol-17 β was purchased from Mann Research Laboratory, New York, N.Y., U.S.A.;

[³H]uridine (29 Ci/mmol), L-[³H]leucine (39 Ci/mmol) and [³H]UTP (40 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.; L-leucine, yeast RNA (type XI) and bovine plasma albumin were from Sigma, St. Louis, Mo., U.S.A.; uridine was from Boehringer, Milan, Italy; Insta-gel was from Packard, Downers Grove, Ill., U.S.A. Other materials were analytical-grade reagents from C. Erba, Milan, Italy.

Measurement of ³H

A three-channel Nuclear-Chicago spectrometer was used. The samples were prepared for radioactivity measurement by mixing with either Insta-gel or toluene scintillator fluid (Puca *et al.*, 1971). Efficiency (about 25%) was determined by the channels-ratio method.

Animals, surgical procedure and treatments

Swiss female mice, 3–4 months old, were used. Ovariectomy was carried out via the dorsal approach, under ether anaesthesia. Chromatographically pure oestradiol-17 β was dissolved in 0.15 M-NaCl-ethanol (2:1, v/v) to the final concentration of 100 mg/litre. Ovariectomized mice were injected intraperitoneally with 0.1 ml of the above oestradiol-17 β solution (10 μ g of hormone/animal).

To measure the incorporation of precursor into RNA and protein *in vivo*, mice were injected intraperitoneally with 0.2 ml of 0.15 M-NaCl containing 30–100 μ Ci of [³H]uridine (sp. radioactivity 10 Ci/mmol), or with 0.2 ml of 0.15 M-NaCl containing 50 μ Ci of L-[³H]leucine (sp. radioactivity 0.8 Ci/mmol). The mice were killed by cervical dislocation 30 min after [³H]uridine injection or 15 min after L-[³H]leucine injection.

Uptake of [³H]uridine by mammary gland

After injection of [³H]uridine and killing after 30 min, the fifth pair of mammary glands (inguinal) were rapidly dissected. The fifth pair were chosen because they are the most easily identified and dissected. After removal of lymph nodes, the glands were frozen (–20°C) before further treatment. Glands from mice which had undergone identical treatments were pooled together. After being thawed, the pooled glands were minced with scissors and homogenized in an Ultra-Turrax homogenizer at 90 V in four bursts lasting 20 s each, with 60 s intervals between bursts. Homogenization was carried out in 3 ml of 1 M-uridine in 0.01 M-NaCl, with the homogenate container immersed in crushed ice.

To measure total radioactivity, 0.1 ml of the homogenate was added to 2.9 ml of water and mixed with 6 ml of Insta-gel.

Incorporation of [³H]uridine into mammary gland, liver and muscle RNA

The mammary-gland homogenate prepared as described above was subjected to the following procedure. Ice-cold acetone (16 ml) was added and, after vigorous shaking, the mixture was centrifuged; the pellet was resuspended in acetone and the procedure repeated once more. The pellet after acetone extraction was resuspended in 16 ml of ice-cold 5% (w/v) trichloroacetic acid containing 0.01 M-NaCl and 1 mM-uridine; after 30 min in the cold, the suspended material was sedimented by centrifugation; the procedure was repeated twice more. Thereafter the pellet was resuspended in 16 ml of ether-ethanol (1:3, v/v), centrifuged and the procedure repeated once more. The final pellet was resuspended in 2 ml of 1 M-KOH and the mixture incubated at 37°C for 20 h in a stoppered vial to hydrolyse RNA. Thereafter, 0.4 ml of 6 M-HCl and 2 ml of 5% (w/v) trichloroacetic acid were added to the KOH hydrolysate and the precipitate was sedimented by centrifugation at 1000 g. The clear supernatant was neutralized with KOH. A portion of the supernatant was mixed with Insta-gel and used for radioactivity measurement; another portion was used for measuring RNA content by the method of Ceriotti (1955), with yeast RNA being used for standard curves. The specific radioactivity of RNA could thus be computed.

The same procedure was used for liver and quadriceps femoris, about 500 mg of each tissue being used as a starting material.

Nuclear RNA synthesis in vitro

Nuclei were isolated by the following procedure. Inguinal mammary glands from groups of 20 mice were dissected and the lymph nodes removed. The glands were cooled, pooled, minced and homogenized in an Ultra-Turrax homogenizer at 70 V in four bursts lasting 15 s each, with 60 s intervals between bursts. Homogenization was performed in 20 ml of ice-cold 0.25 M-sucrose containing 3 mM-MgCl₂ and 1 mM-NaHSO₃. The homogenate was filtered twice through one layer of cheesecloth and centrifuged for 15 min at 800 g. The pellet was suspended in 20 ml of homogenizing medium and centrifuged again. The final pellet was suspended in 2 ml of 0.05 M-Tris-HCl, pH 7.9, containing 1 mM-MgCl₂ and 25% (v/v) glycerol. A sample of the suspension was used for DNA measurement (Giles & Myers, 1965), and stored for no more than 2 days at –20°C before assessment of RNA synthesis by the procedure of Glasser *et al.* (1972), modified as follows: 1 ml of low-salt reaction mixture included 50 μ mol of Tris-HCl, pH 7.9, 1.6 μ mol of MnCl₂, 2 μ mol of MgCl₂, 8 μ mol of KCl, 6 μ mol of NaF, 1 μ mol of dithiothreitol, 0.6 μ mol each of CTP, GTP and ATP, 25 μ Ci of [³H]UTP (about 40 Ci/mmol) and 4 μ g of α -amanitin. High-salt reac-

tion mixture was as described above except that α -amanitin was omitted and 250 μ mol of $(\text{NH}_4)_2\text{SO}_4$ was included. The reaction was started by adding nuclei (40 μ g of DNA/ml), and incubation lasted 10 min at 15°C.

The reaction was terminated by adding 0.7 ml of sample to 1 ml of 10% (w/v) trichloroacetic acid. After shaking, the precipitates were collected on Millipore filters (0.45 μ m pore size) and washed four times with 10 ml of 5% trichloroacetic acid containing 1% (w/v) $\text{Na}_4\text{P}_2\text{O}_7$. Filters were then dried, washed twice with 5 ml of toluene and radioactivity was counted in toluene scintillator fluid. Thereafter the filters were dried again, and incubated for 30 min at 90°C in 2 ml of 5% (w/v) trichloroacetic acid. The DNA content of 1.5 ml of the trichloroacetic acid hydrolysate was assayed by the method of Giles & Myers (1965).

Incorporation of L-[^3H]leucine into total mammary-gland protein

Animals were killed 15 min after L-[^3H]leucine injection. Incorporation into proteins of the mammary gland continues for at least 1 h after injection of labelled amino acids (Lavrin, 1963). Mammary glands were subjected to the same procedure as was used for measurement of [^3H]uridine incorporation into RNA. Trichloroacetic acid was added to the homogenate (to 5% final concentration), which was then heated at 90°C for 15 min and centrifuged. The pellet was washed with 50 and 70% ethanol and then dissolved in 1.3 ml of 10 mM-NaOH. A portion of the dissolved material was mixed with Insta-gel and used for radioactivity measurement; another portion was used for assessing protein content by a modified Lowry procedure (Eggstein & Kreutz, 1955), with crystalline bovine plasma albumin as standard.

Results

As shown in Fig. 1, the ability of the mammary gland of adult mice to incorporate [^3H]uridine into RNA decreases as a function of time after ovariectomy. At 3 weeks after removal of the ovaries, the decrease in rate of precursor uptake into RNA is very substantial (residual activity is about 18% of control), whereas incorporation of L-[^3H]leucine into protein is only slightly affected (residual activity is about 90% of control). The weight of the gland is not significantly changed.

A single intraperitoneal injection of oestradiol-17 β into these ovariectomized mice (10 μ g per animal) results in early stimulation of the rate of precursor uptake into total RNA of mammary gland (Fig. 2a). The increase is already significant ($P < 0.05$) 15 min after hormone administration, the earliest time-point in the study, as well as at 30, 60, 90 and 120 min. After

Fig. 1. Effect of ovariectomy on wet weight (□) and incorporation of radioactive precursor into mammary-gland RNA (○) and protein (Δ) *in vivo*

Adult ovariectomized mice were injected with either 100 μ Ci of [^3H]uridine 30 min before death, or 50 μ Ci of L-[^3H]leucine 15 min before death. At the times indicated, animals were killed in groups of three and their inguinal mammary glands (fifth pair) excised, weighed and pooled for homogenization and further treatment. Specific radioactivity of RNA and protein was determined in the gland homogenate. Data are expressed as percentage of control, i.e. mammary glands of mice killed immediately after ovariectomy (zero time). The following control values were obtained: wet weight of six glands, 864 mg; specific radioactivity of RNA, 57000 d.p.m./mg; specific radioactivity of protein, 5700 d.p.m./mg.

the first burst, the incorporation rate returns to the pre-stimulation value, with no significant difference ($P > 0.05$) from the basal value being detected at 3, 4, 6, 7 and 8 h after hormone injection. A second burst of increased activity shows a significant peak at 9 h after oestradiol-17 β injection, a third at 12 h and a fourth at 16 h. The peaks are separated by periods in which precursor uptake decreases to about pre-stimulation values, with no significant difference from basal values detected at 10, 11, 13–15 and 17 h after hormone injection. The qualitative occurrence of these peaks is remarkably reproducible. Significance of differences from value at zero time was tested according to the 'null hypothesis'.

Incorporation of [^3H]uridine into total RNA of liver and quadriceps muscle of mice injected with oestradiol-17 β was measured in two different experiments (Figs. 2b and 2c). No significant stimulation of the precursor uptake into RNA of these two non-target organs was observed after hormonal injection.

As shown in Fig. 3 for the first burst of increased incorporation into RNA, the total uptake of [^3H]uridine into the gland shows only a barely detectable

increase during the oestrogen-induced periods of highly augmented incorporation into RNA. Also, the weight of the gland is not affected. An increase in total uptake of at most 1.2-fold corresponds to a

Fig. 2. Effect of a single injection of oestradiol-17 β on [^3H]uridine incorporation into RNA of mammary gland (a), liver (b) and quadriceps muscle (c) of ovariectomized adult mice

Adult mice 3 weeks after ovariectomy were used. In the experiments reported in (a) mice were given 10 μg of oestradiol-17 β intraperitoneally and injected with 100 μCi of [^3H]uridine 30 min before death. At the time indicated, animals were killed in groups of three and both their inguinal mammary glands (fifth pair) excised and pooled for homogenization and further treatment. Numbers of animals at each time-point were as follows: at time 0, 27 animals; at times 0.25, 0.5, 7, 11–17 h, 12 animals; at times 1, 8–10 h, 18 animals; at times 1.5 and 3 h, nine animals; at time 2 h, 15 animals; at times 4–6 h, six animals. Specific radioactivity of RNA was determined in the gland homogenate. Experimental points are average values. Standard error is shown only when the average is the result of at least three different measures of specific radioactivity, each on a different pool of three mice (six glands). Experiments reported in (b) and (c) are identical with those in (a), except for the amount of [^3H]uridine injected (30 μCi) and for the tissue examined. Experimental points in (b) and (c) are averages of two different experiments.

fivefold increase in specific radioactivity of RNA. These results make it unlikely that increased specific radioactivity of RNA can be accounted for by an increase in the specific radioactivity of the precursor pool resulting from higher intracellular concentration of [^3H]uridine.

Further evidence that the observed increase in the uptake of [^3H]uridine into RNA of oestrogen-stimulated mammary glands truly reflects an increased transcription was gained by studying RNA synthesis by isolated nuclei *in vitro*. Nuclei were prepared from mammary-gland homogenates by a method which yields as a routine a nuclear preparation containing about 60% of the DNA in the original homogenate, with a DNA/protein ratio of 0.104, compared with 0.017 for the homogenate. Nuclei are thus purified about sixfold. [^3H]UTP is incorporated linearly into the RNA of this nuclear preparation with time for at least 20 min, in either the absence or the presence of 0.25 M-(NH_4) $_2\text{SO}_4$. The activity in the presence of (NH_4) $_2\text{SO}_4$ is about four times that in its absence. [^3H]UTP incorporation depends on the presence of all four nucleotides and is totally inhibited by 20 μg of actinomycin D/ml. As shown in Fig. 4, in the presence of (NH_4) $_2\text{SO}_4$ (high salt) the incorporation is strongly inhibited by α -amanitin, with

Fig. 3. Effect of a single injection of oestradiol-17 β on wet weight (Δ), total [^3H]uridine uptake ($\square$) and specific radioactivity of RNA ($\circ$) of mammary gland of ovariectomized adult mice

Experimental procedures are as described in the legend to Fig. 2, except that mammary glands were carefully dissected and weighed before further processing. Total radioactivity was referred to g of fresh tissue. Data are expressed as percentages of control values. The control values are as follows: wet weight of six glands, 874 mg; total radioactivity, 785 300 d.p.m./g wet wt.; specific radioactivity of RNA, 11 600 d.p.m./mg.

Fig. 4. Effect of α -amanitin on RNA synthesis *in vitro* by mammary-gland nuclei from ovariectomized mice

The activity was measured in high-($\circ$) and low-($\bullet$) salt conditions (see the Experimental section). Control values of the radioactivity were 4000 and 750 c.p.m./100 μg of DNA respectively.

Fig. 5. Effect of a single injection of oestradiol-17 β *in vivo* on [^3H]uridine incorporation *in vitro* into RNA of nuclei from mammary glands

Animals were injected with 10 μg of hormone and killed in groups of 20 at the times indicated. Mammary-gland nuclei were isolated, and RNA synthesis was measured in triplicate, at 15°C, either in the absence of $(\text{NH}_4)_2\text{SO}_4$ and in the presence of 4 μg of α -amanitin (open bar) or in the presence of 250 mM- $(\text{NH}_4)_2\text{SO}_4$ (shaded bar). Data are averages from two or three different experiments (standard deviations are less than $\pm 10\%$) and expressed as percentage of control, i.e. mammary glands from mice killed immediately after oestradiol-17 β injection. Control values are 800 and 4000 c.p.m./100 μg of DNA for the radioactivities in low- and high-salt conditions respectively.

80% inhibition being reached at 4 $\mu\text{g/ml}$ or more. Thus apparently 80% of the activity measured under high-salt conditions is due to polymerase II (Lindell *et al.*, 1970). In the absence of $(\text{NH}_4)_2\text{SO}_4$ (low salt),

4 μg of α -amanitin/ml inhibits only about 20% of incorporation, and therefore under such conditions most of the activity measured appears to be RNA polymerase I. Thus 4 μg of α -amanitin/ml was the concentration subsequently used in all assays performed in low-salt conditions.

Fig. 5 shows the results of [^3H]UTP incorporation into RNA of isolated nuclei from mammary glands of ovariectomized mice and of ovariectomized mice injected with oestradiol-17 β either 30 min, 1 h, 4 h or 12 h before death. Both RNA polymerase activities show an increase at 30 min, and even more at 1 h after oestradiol administration; the activities decline at 4 h and increase again at 12 h. These results in general confirm those obtained by measuring [^3H]uridine incorporation into total RNA directly in the animal and suggest that the oestrogen effect is truly a stimulation of the transcription mechanism.

Discussion

The results show that ovariectomy has a drastic effect on [^3H]uridine incorporation into RNA of mammary gland, which decreases to about 20% of original activity within 21 days. A single injection of oestradiol-17 β (10 μg /mouse) at this point has a rapid stimulatory effect on [^3H]uridine incorporation into RNA, which is already detectable at 15 min (the earliest time-point investigated) and reaches a maximum at 60 min after the hormone injection. Thereafter, despite the continued presence of active concentrations of hormone in the gland (Puca & Bresciani, 1969), the activity decreases again to reach the pre-stimulation value at about 4 h. Subsequently, the activity undergoes further undulatory changes, with peaks at 9, 12 and 16 h after hormone injection. The above polyphasic response to oestradiol was shown to be statistically significant. There is no significant effect on [^3H]uridine uptake into RNA of quadriceps femoris and liver. Evidence that the above changes in [^3H]uridine incorporation into RNA are not a consequence of pool changes was obtained by investigating [^3H]UTP incorporation into RNA of isolated nuclei from glands of ovariectomized oestradiol-injected mice. We tested the early increase (30 min and 60 min), the decline at 4 h and the peak at 12 h by the above method, to find a good correspondence to experiments *in vivo* with total glands. Both polymerases I and II appear to be responsible for the increased incorporation of precursor into RNA.

Additional indications that the changes in [^3H]uridine uptake are not pool-dependent can be found in the fact that the total weight of the mammary gland does not change significantly after oestradiol injection, and total uptake of [^3H]uridine per unit weight of the gland is virtually unchanged or is at most increased by about 20% when precursor incorpora-

tion into RNA is increased about fivefold; such a slight increase in total precursor uptake cannot justify the much higher specific radioactivity of RNA and, in all likelihood, is the consequence and not the cause of enhanced precursor incorporation into RNA.

In hormone-dependent rat mammary tumours a single injection of oestradiol also strongly stimulates [^3H]uridine incorporation into RNA within a few minutes of injection of the hormone. Similarly, as in the present case, early stimulation is transitory; within 1 h of injection of the hormone uridine incorporation returns to the pre-stimulation value and stays there for the next 6 h, which is the entire time-span covered in the study of Gullino *et al.* (1974). Also, in rat uterus a burst of increased [^3H]uridine incorporation into RNA occurs within a few minutes of injection of the hormone (see reviews by Tata, 1966; Hamilton, 1968, 1971); thereafter the incorporation decreases to about twice the pre-stimulation value within 2 h of injection of the hormone; two measurements at 12 and 24 h after stimulation show about the same rate of incorporation as observed at 2 h.

Therefore, although an early burst of precursor uptake into RNA appears to be a common feature of target tissues for oestradiol, there is a difference between normal and neoplastic mammary-gland tissues on the one hand and uterus on the other, in that in the former there is a return to pre-stimulation values, whereas in the latter the value seems to remain always higher. Only the present study follows at short intervals the behaviour of RNA synthesis after the initial burst, a necessary requirement to avoid missing the rather sharp changes in the rate of synthesis which occur after the earliest one.

Some general comments on the observed polyphasic response of RNA synthesis to oestrogen stimulation appear justified. First, we know that the present artificial conditions of oestrogen stimulation induce responsive cells to pass synchronously through DNA synthesis and mitosis (Bresciani, 1971). In the intact untreated animal asynchrony is rather the rule and therefore cells are randomly distributed in different stages of the process. Only when cells are synchronous, therefore, is it experimentally possible to detect sequences of discrete phenomena.

Finally, there is the intriguing question of the biological significance of the polyphasic response. We

suggest as a working hypothesis that the series of discrete subsequent bursts of RNA synthesis that is seen within the initial hours of oestrogen injection, is the orderly expression of a programme incised in the DNA and which is specifically activated by oestrogen. Development of this programme will eventually lead to DNA synthesis and cell replication as well as to the other phenomena of the complete oestrogen effect in the mammary gland.

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References

- Bresciani, F. (1964) *Science* **146**, 653–655
- Bresciani, F. (1965) *Exp. Cell Res.* **38**, 13–26
- Bresciani, F. (1968) *Cell Tissue Kinet.* **1**, 51–63
- Bresciani, F. (1971) in *Basic Actions of Sex Steroids on Target Organs* (Hubinot, P. O., Leroy, F. & Galand, P., eds.), pp. 130–159, S. Karger, Basel and New York
- Bresciani, F. & Puca, G. A. (1965) *Atti Soc. Ital. Patol.* **9**, 661–664
- Bresciani, F., Puca, G. A., Nola, E., Salvatore, M. & Ardovino, I. (1969) *Atti Soc. Ital. Patol.* **11**, 203–224
- Cerioti, G. (1955) *J. Biol. Chem.* **214**, 59–71
- Eggstein, M. & Kreutz, F. H. (1955) *Klin. Wochenschr.* **33**, 879–881
- Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93
- Glasser, S. R., Chytil, F. & Spelsberg, T. C. (1972) *Biochem. J.* **130**, 947–957
- Gullino, M., Cho Chung, Y. S., Losonczy, I. & Grantham, F. H. (1974) *Cancer Res.* **34**, 751–757
- Hamilton, T. H. (1968) *Science* **161**, 649–661
- Hamilton, T. H. (1971) in *Basic Actions of Sex Steroids on Target Organs* (Hubinot, P. O., Leroy, F. & Galand, P., eds.), pp. 56–77, S. Karger, Basel and New York
- Lavrin, D. H. (1963) Ph.D. Thesis, University of California
- Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G. & Rutter, W. J. (1970) *Science* **170**, 447–449
- Puca, G. A. & Bresciani, F. (1969) *Endocrinology* **85**, 1–10
- Puca, G. A., Orii, H. & Fragomele, L. (1967) *Atti Soc. Ital. Patol.* **10**, 979–983
- Puca, G. A., Nola, E., Sica, V. & Bresciani, F. (1971) *Biochemistry* **10**, 3769–3780
- Shyamala, G. & Nandi, S. (1972) *Endocrinology* **91**, 861–867
- Tata, J. R. (1966) *Prog. Nucleic Acid Res. Mol. Biol.* **5**, 191–250