INHIBITION BY α-AMANITIN OF THE OESTRADIOL-INDUCED INCREASE IN α-AMANITIN INSENSITIVE RNA POLYMERASE IN IMMATURE RAT UTERUS

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1. Introduction

The injection of oestradiol to the prepuberal or castrated rat results, 2–3 hr later, in a large increase in RNA biosynthesis [1] and in an increased ability of uterine nuclei to incorporate nucleoside triphosphates into RNA [2]. Various reports have established that oestrogen-stimulated RNA is largely of ribosomal nature [3, 4]. Recently, two different classes of DNA-dependent RNA polymerases have been solubilized and separated from rat liver and calf thymus [5, 6]: one is of nucleolar origin (enzymes A or I) and insensitive to α-amanitin [10], the other is mainly nucleoplasmic (enzymes B_I, B_{II} or II) and inhibited by α-amanitin [7, 10]. It has been shown that 2 hr after injection of oestradiol to immature female rats, the activity of polymerase A, which is unaffected by α-amanitin, increases. This enzyme catalyses the synthesis of ribosomal-type RNA and may be assayed in a medium of low ionic strength [11].

The administration of inhibitors of protein synthesis, such as puromycin or cycloheximide, just before or even immediately after oestradiol injection suppresses the increased RNA synthesis by low salt RNA polymerase activity [12]. It thus appears that one of the earliest effects of oestradiol is to induce a "first-stage" protein synthesis, sufficiently slight to pass easily undetected by conventional techniques during the "lag period" of the first hour, but of crucial importance for the development of most, if not all, the subsequent aspects of oestradiol action including increased RNA synthesis observed after 1–3 hr. Moreover, some experiments [13] seem to imply that continuous protein synthesis is necessary to maintain the oestradiol-induced increase in RNA polymerase activity. On injection of rats with oestradiol and excision of the uterus 2–3 hr later, low salt RNA polymerase activity is found to have increased in the treated uteri as compared to the controls and a significant difference in activity may be maintained for 2 hr if the uteri are incubated at 37° in Eagle's medium. Cycloheximide in the incubation medium suppresses that part of RNA polymerase activity due to oestradiol, thus implying that some activating protein(s) continue(s) to be synthesized in the surviving uteri.

Since it appears that the oestrogen stimulation of RNA polymerase activity requires protein synthesis, it would be interesting to know whether or not this synthesis is promoted at the translational level. Addition of Actinomycin D to the culture medium, in which the uteri are incubated in the experiments just mentioned above, would very likely inhibit RNA polymerase activity in subsequently tested isolated nuclei, but it would not be possible to deduce whether this would be the consequence of inhibition of the end reaction (increased ribosomal RNA polymerase activity) or whether an initial RNA synthesis catalysed by extranucleolar polymerase would be necessary for the synthesis of polymerase activity protein. However, the need for a "first stage" RNA synthesis can be estab-

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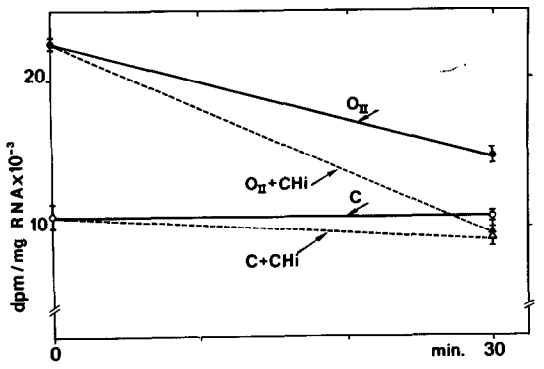

Fig. 1. Nuclear RNA polymerase activity in oestradiol-primed (O_{II}) and control (C) uteri. Incubation at 37°; CHi: cycloheximide in the incubation medium.

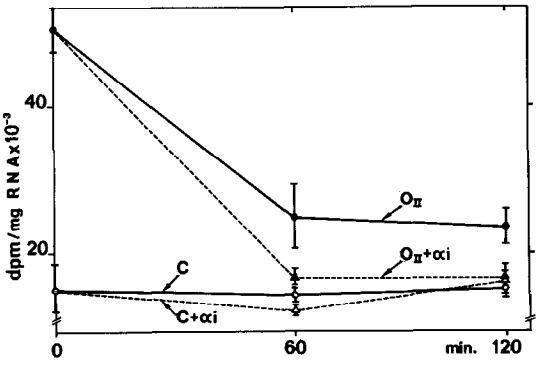

Fig. 2. Nuclear RNA polymerase activity in oestradiol-primed (O_{II}) and control (C) uteri. Incubation at 37°; αi: α-amanitin in the incubation medium.

lished by the use of a specific inhibitor: the fungal polypeptide α-amanitin which, however, does not block the secondarily increased polymerase activity. The effect of α-amanitin, added during the incubation of uteri, on the RNA polymerase activity of isolated nuclei obtained either from control animals or animals treated with oestradiol, was therefore investigated.

2. Methods

Oestradiol (5 μg dissolved in 0.5 ml of a 0.9% NaCl, 5% ethanol solution) was administered subcutaneously to immature (20–23 day old) female Sprague Dawley rats. Control animals were injected with solvent only. Three hr after the injection, the animals were decapitated. Their uteri were excised and either assayed immediately for RNA polymerase activity (zero time on figures) or placed into flasks containing 2 ml of Eagle modified Dulbecco medium at 37°. Flasks, containing 4 slit uterine horns each, were incubated in a shaking waterbath at 37° in an atmosphere of 95% O_2 –5% CO_2 for periods of time varying between 0–2 hr. Where indicated, α-amanitin (1 μg/ml: 1.2 μM) was added to the incubation medium.

Following incubation, the uteri were homogenized. A nuclear preparation was obtained and the RNA polymerase assayed [2]. The nuclear suspension from the uteri of each group was divided equally for 2–5 determinations. 0.3 ml of nuclear suspension, corresponding to approx. 200 μg of DNA, was incubated at 37° in 1 ml of medium containing ^{3}H-CTP, either with or without α-amanitin. Since the incorporation of ^{3}H-CTP reaches a plateau after 10–15 min [14], 20 min were chosen for single experimental points under the various conditions. After chilling, 1 ml of cold 10% $HClO_4$ was added. The contents of the tubes were centrifuged and the precipitate was washed 3 times with 5% $HClO_4$, twice with ethanol, then dried. The RNA was hydrolyzed with 0.3 N KOH for 1 hr at 37°. The dpm incorporated per mg of RNA were determined as described previously [11].

3. Results

The activity of low salt DNA-dependent RNA polymerase in the rat uterus rises rapidly after *in vivo* treatment wtih oestradiol ([2], see figures : zero time ordinates). The oestradiol-stimulated increase in RNA synthesis in uterus nuclei prepared immediately after decapitation decreases significantly if the uteri have previously been incubated in Eagle's medium. However, a difference in the level of the polymerase activity between the oestrogen-treated and control uteri is maintained for 2 hr if the surviving uteri have been incubated at 37° in tissue culture medium (solid lines on all figures). The addition of cycloheximide (25 μg/ml) to the incubation medium suppresses that part of RNA polymerase activity due to oestradiol. This result suggests that activating protein is synthesized in the surviving uterine horns, and

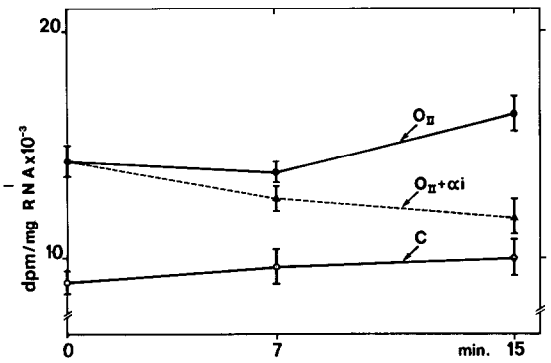

Fig. 3. Nuclear RNA polymerase activity in oestradiol-primed (O_{II}) and control (C) uteri. Incubation at 37°; αi: α-amanitin in the incubation medium.

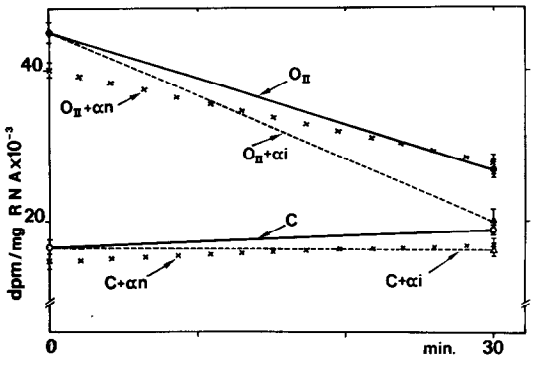

Fig. 4. Nuclear RNA polymerase activity in oestradiol-primed (O_{II}) and control (C) uteri. Incubation at 37°; αi: α-amanitin in the incubation medium; αn: α-amanitin during nuclear RNA polymerase assay.

confirms the results of Nicolette and Mueller [13]. A dynamic study reveals that the increase due to oestradiol was suppressed already after 30 min (fig. 1, O_{II}-vs-O_{II} + CHi), thus indicating that the apparent half-life of the protein is approximately 15 min under these conditions.

The oestrogen-induced component of RNA polymerase activity is furthermore very sensitive to the action of α-amanitin: 1 μg/ml of this agent in the tissue culture medium reduced within 1 hr the RNA polymerase activity of surviving oestrogen-treated uteri to the level of that of the control uteri (fig. 2). On the other hand, the same concentration of α-amanitin in the culture medium has almost no effect on the polymerase activity of control uteri. These experiments suggest that the maintainance of the oestrogen-induced fraction of uterine polymerase activity requires the concomitant synthesis of RNA.

The very short half-life of RNA is illustrated in fig. 3. Control and oestradiol-treated uteri are incubated for a very short time, 7 or 15 min in the culture medium with or without α-amanitin. The presence of α-amanitin in the incubation medium gives rise to marked inhibition: 70% of the oestradiol-induced increase in RNA polymerase activity within 15 min.

To counteract the oestrogen stimulation, α-amanitin has to be present during the *in vitro* incubation of the surviving uteri, as it has no effect when added directly to the low salt nuclear system used to assay RNA polymerase [11]. Indeed, α-amanitin has only a very limited effect on the low-salt RNA polymerase activity of control nuclei or oestradiol-treated nuclei previously incubated in the culture medium (fig. 4). This confirms, therefore, that the inhibition of the oestrogen-induced fraction of uterine polymerase activity by α-amanitin occurs only when it is added to the incubation medium prior to the preparation of the nuclear fraction and RNA polymerase assay.

4. Discussion

Earlier studies have shown that α-amanitin insensitive nucleolar RNA polymerase activity increases 2 hr after the injection of oestradiol to immature female rats [11]. On increasing the ionic strength of the nuclear RNA polymerase assay medium, this increase is suppressed, thus suggesting the presence of an oestradiol-induced component which activates α-amanitin resistant RNA synthesis, but is inactive in high salt medium. The component could be a new α-amanitin resistant polymerase or other factor, e.g. a protein interacting with a polymerase or some other component of the chromatin machinery involved in α-amanitin resistant RNA synthesis, and unable to do so in a medium of high ionic strength [11].

The present experiments indicate that the oestradiol-induced increase in low salt RNA polymerase activity is sensitive to protein synthesis inhibitors and to α-amanitin. It thus appears that a protein activating

α-amanitin insensitive polymerase activity may be formed which is itself dependent on a short-lived RNA synthesized by α-amanitin sensitive polymerase and therefore presumably mRNA. Another, and rather similar, inhibition of the synthesis of ribosomal RNA in rat liver nuclei follows the *in vivo* administration of α-amanitin [15]. One explanation for this could be that the action of the ribosomal RNA polymerase is regulated by some extranucleolar factor sensitive to α-amanitin.

It is thus possible that some type of RNA formed in the extranucleolar compartment exerts a regulatory effect over rRNA formation. Protein factors stimulating the activity of mammalian RNA polymerase have indeed been identified for extranucleolar polymerase B in calf thymus [16] and rat liver [17]. It is therefore quite conceivable that factors which stimulate nucleolar polymerase A activity might exist.

The synthesis of such an activating protein which in turn stimulates α-amanitin resistant, presumably nucleolar, RNA polymerase activity may be a decisive and possibly also limiting step in hormone action. Since no early increased synthesis of mRNA has been demonstrated for the various proteins involved in tissue growth, the increase in polymerase activity involved in the synthesis of other RNAs (for example rRNA) could be a key mechanism in providing more protein synthesis by increased used of the mRNAs (for example, by increased processing transport, or translation). In this case, the activating protein would be a Key Intermediary Protein (KIP) which it would be very important to study. *In vivo* and *in vitro* experiments giving direct evidence for KIP and its potential mRNA are described elsewhere [18, 19].

Acknowledgements

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