A ROLE FOR DNA IN THE FORMATION OF NUCLEAR ESTRADIOL-RECEPTOR COMPLEX IN A CELL-FREE SYSTEM.

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Summary - Destruction of the DNA component of the nuclear pellet fraction of rat uterus homogenates with DNase prevented the formation of "nuclear" estradiol-receptor complex upon subsequent incubation with supernatant fraction containing 8S cytoplasmic estrogen-receptor complex. Nuclear pellet pretreatment with RNase had no effect on the reaction. DNase did not appear to release any component capable of binding estradiol or the 8S estradiol-receptor complex.

When the immature rat uterus is exposed to estradiol-^{3}H *in vivo* or *in vitro*, hormone taken up by target cells first appears as a complex with a cytoplasmic receptor protein, sedimenting at 8S on sucrose gradients (1). The hormone-receptor complex subsequently migrates to the cell nucleus (2,3,4,5), from which an estrogen-receptor complex sedimenting at 5S, apparently deriving from the 8S complex (4,6), can then be extracted with solutions of 0.4 M KCl.

The formation of nuclear 5S complex can be duplicated in a cell-free system in which the nuclear pellet fraction is incubated with supernatant fraction containing estradiol. The reaction specifically requires the presence of 8S cytoplasmic complex, and does not occur with nuclear fractions from non-target tissues such as diaphragm (4,5). KCl extracts in this system contain a 4S complex as well as 5S complex. The 4S complex

is presumed to represent a subunit released from the 8S complex in the presence of high concentrations of salt (4,5,7,8).

The formation of nuclear complexes in this system has been shown to depend on a heat-labile intranuclear factor (5). In order to further distinguish what nuclear components are involved in this process, the effects of nuclease pretreatment of nuclear pellet fraction have been studied. It has been found that the destruction of endogenous nuclear DNA by DNase interferes with subsequent formation of nuclear 4 and 5S complex.

Materials and Methods: Estradiol-6,7-^{3}H (specific activity 38 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass.. DNase I and RNase A were purchased from Worthington Biochem. Corp., Freehold, N.J.. Actinomycin D was a gift of Merck, Sharpe and Dohme.

Cell-free supernatant and "nuclear pellet" fractions were prepared from uterine horns from 21 to 24 day old rats, by homogenization and centrifugation as described previously (5). Pretreatment of pellet fractions with nuclease was performed by rehomogenizing material from 7 uterine horns in 0.8 ml of tris-EDTA buffer (0.01 M tris, 0.0015 M EDTA, pH 7.4), tris buffer (0.01 M tris, pH 7.4) or tris-Mg^{2+} buffer (0.01 M tris, 0.005 M $MgCl_2$, pH 7.4), containing DNase I (500 ug/ml), RNase A (500 ug/ml), or no enzyme, as indicated, and incubating for 20 minutes at 25°. Pellet fraction was then re-isolated by centrifugation for 30 minutes at 45,000 rpm in a Spinco Model L2-65B ultracentrifuge.

Incubations of cell-free fractions utilized material from 7 uterine horns homogenized in 0.5 ml of tris-EDTA buffer. Pellet fractions were rehomogenized with supernatant containing 3×10^{-8}M estradiol-^{3}H, incubated for 10 minutes at 37°, and then again separated by ultracentrifugation. Nuclear estradiol-^{3}H-

receptor complexes were extracted from the pellet fractions with 0.4 M KCl in tris-EDTA buffer and analyzed on 5-20% sucrose gradients as previously described (5).

In experiments designed to detect release of an 8S complex binding component from the pellet fraction by DNase, 0.15 ml of supernatant fraction containing cytoplasmic 8S complex was mixed with an equal volume of the supernatant of pellet fractions pre-incubated in tris-Mg^{2+} buffer with or without DNase as described above. These mixtures were incubated for 30 minutes at 0^{o} or 5 minutes at 37^{o} and then analyzed on 5-20% sucrose gradients (5).

Results: Uterine nuclear pellet fractions which had been pretreated with DNase or RNase were compared to pellets pre-incubated in buffer without enzyme for their ability to form 4S and 5S nuclear estradiol-receptor complexes upon incubation with supernatant containing 8S complex. The results are shown in Figure 1. While RNase pretreatment had no effect on subsequent 4S and 5S complex formation, DNase abolished the 5S peak and markedly diminished the 4S peak normally found in 0.4 M KCl extracts of pellet incubated with 8S complex. Total accumulation of estradiol by the pellet fraction, as well as total depletion of 8S complex from the supernatant fraction, both shown previously to reflect in large measure non-specific processes (5), were little affected by DNase pretreatment.

Separate experiments showed that DNase alone had no effect on the sedimentation profiles of 8S complex, or 4S and 5S complex isolated from pellets not treated with DNase. DNase pretreatment of pellet fractions in the absence of Mg^{2+} ion caused a considerably smaller or often undetectable decrease in 4S and 5S complex. The presence of 0.005 M $MgCl_2$ during the pretreatment of pellet fractions did not alone affect the production of nuclear complexes

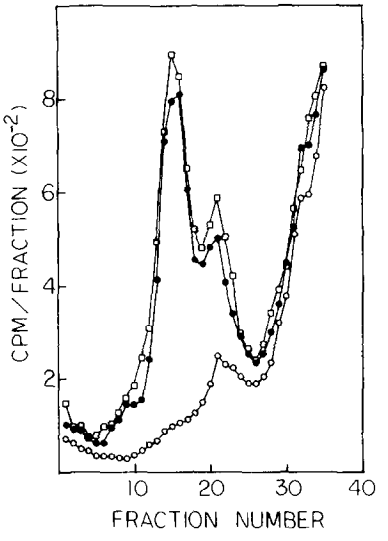

Figure 1. Nuclear complex formation in pellet fractions pretreated with DNase or RNase. Pellet fractions from 7 uterine horns were homogenized in 0.8 ml of a) tris-Mg^{2+} buffer with no added enzyme (●——●) b) tris-Mg^{2+} buffer with DNase I, 500 ug/ml, (o——o) or c) tris-EDTA buffer with RNase, 500 ug/mg, (□——□) and incubated 20 min. at 25°. Pellets were then isolated by centrifugation and rehomogenized in 0.5 ml of supernatant fraction from 7 uterine horns containing 3 x 10^{-8}M ^{3}H-estradiol and incubated for 10 min. at 37°. Pellet fractions were again isolated and extracted with 0.5 ml of 0.4 M KCl buffer. Aliquots of 0.2 ml of the KCl extracts were layered on 5-20% sucrose gradients and centrifuged for 17 hours at 190,000 x g.

in subsequent cell-free incubation in tris-EDTA buffer.

The presence of actinomycin D in concentrations of 1 to 10 ug/ml during the incubation of supernatant with non-pretreated pellet fractions did not diminish the formation of 4S and 5S nuclear complexes.

To evaluate the possibility that DNase pretreatment might release a component from the nuclear pellet capable of binding estradiol or the 8S estradiol-receptor, the soluble fractions of pellet homogenates pretreated with or without DNase were isolated by centrifugation, mixed with fresh supernatant containing 8S complex, incubated at 0° or 37°, and analyzed on 5-20% sucrose

gradients. The soluble fraction from DNase-treated pellet did not reduce the amount or alter the sedimentation profile of the 8S complex compared to mixtures containing soluble fraction from pellet homogenates without DNase.

Discussion: The properties and specificity of the cell-free system used in these experiments have been described in considerable detail previously (2,5,9). The reaction has been found to be a useful model for studying the in vivo interaction of the cytoplasmic steroid-receptor complex with the cell nucleus. Destruction of the DNA component of the nuclear pellet fraction by DNase has been shown here to prevent the subsequent appearance of nuclear complexes upon incubation with supernatant containing 8S complex. RNase pretreatment had no effect on the reaction.

In interpreting this result, the possibility must be considered that DNase might non-specifically react with either the cytoplasmic receptor or its nuclear "acceptor" binding site. However, the fact that the bulk of the soluble DNase is separated from the system prior to incubation of pellet with supernatant fraction, the absence of any effect of DNase on the sedimentation profiles of 8S, 4S, or 5S complex, and the fact that DNase has its effect only under conditions that favor its hydrolytic activity (i.e. in the presence of Mg^{2+}), minimize the possibility of a non-specific interaction. The fact that actinomycin D does not interfere with the formation of nuclear complex also excludes the possibility that DNase has its effect simply by blocking DNA-dependent RNA synthesis.

It is not clear at present if the dependence of 5S complex formation on nuclear DNA results from a direct role of the DNA in the reaction, presumably binding of 8S, 5S or 4S complex. It is also possible that the DNA plays an indirect role; the hormone-

receptor might interact with a nuclear component associated with the DNA, which with the destruction of the DNA, is released or changed so as to become unreactive or inaccessible. Although the latter possibility has not been excluded, experiments to be reported elsewhere (10) indicate that the cytoplasmic steroid-receptor complex binds to purified double stranded DNA *in vitro*, lending support to the alternative of direct DNA involvement. Furthermore, no component capable of binding to 8S cytoplasmic complex could be detected in the soluble fraction of pellet homogenates pre-incubated with DNase, by sucrose gradient centrifugation.

The nuclear "acceptor" site for the cytoplasmic estradiol-receptor complex, therefore, may be the DNA itself. Other nuclear components (*e.g.* acidic proteins or histones) might play a role in specifying the site or extent of binding, or participate in the processing of cytoplasmic complex and formation of nuclear complex. Alternatively, the acceptor site may be another component of the chromatin, which depends for its ability to bind cytoplasmic receptor or form nuclear complex on the integrity of the nuclear DNA.

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