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INHIBITION OF THE ESTROGEN-INDUCED SYNTHESIS OF VITELLOGENIN mRNA IN CHICK LIVER BY TAMOXIFEN

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Cloned vitellogenin cDNA (labelled with ^{32}P) was used as a probe for measuring vitellogenin mRNA sequences in RNA preparations from the liver of chicks treated with estradiol and/or tamoxifen. For the first time it was shown that the antiestrogen tamoxifen inhibits the estradiol-induced synthesis of vitellogenin mRNA in chick liver. This inhibition correlates very well with a reduced capacity of the liver to synthesize vitellogenin. Furthermore, evidence is presented that tamoxifen lacks any agonistic activity in chick liver. Vitellogenin mRNA is not measurable after tamoxifen alone.

Recently, we found that antiestrogens, like nafoxidine, CI-628 and tamoxifen, inhibit the estrogen-induced synthesis of vitellogenin in chick liver and that, in contrast to results in rat uterus (1,2), these antiestrogens exhibit no estrogenic activity in chick liver with respect to the induction of vitellogenin synthesis (3-5). Similar results were obtained by another group (6). We now present the first data, which demonstrate that the antiestrogen tamoxifen inhibits the synthesis of mRNA for vitellogenin. Furthermore, we demonstrate the lack of estrogenic potency of tamoxifen in chick liver also at the level of vitellogenin mRNA production.

MATERIALS AND METHODS

Materials: Estradiol-17β (1,3,5(10)-estratriene-3,17β-diol) was from Merck, Darmstadt. Tamoxifen was a gift from ICI Ltd. (Pharmaceuticals Division, Macclesfield, Great Britain).

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Buffers: Buffer A: 120 mM phosphate, pH 6.8, 50% formamide, 0.45 M NaCl, 45 mM sodium citrate, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl-pyrrolidone.
Buffer B: 0.3M NaCl, 30 mM sodium citrate.
Buffer C: 15 mM NaCl, 1.5 mM sodium citrate.

Animals: Newborn chicks were obtained locally and were given a standard diet ad libitum. 5-6-week-old animals were injected i.m. with estradiol and/or tamoxifen dissolved in propylene glycol, as given in the text.

Purification of vitellogenin mRNA ($mRNA_{vit}$). Total RNA was extracted from chick liver by the method of Deeley et al. (7). Poly(A)-RNA was prepared by chromatography of total RNA on poly(U)-Sepharose according to Jost and Pehling (8). After two consecutive sucrose density gradient centrifugations (5-30% and 10-23% sucrose) a partially purified $mRNA_{vit}$ preparation was obtained from the fractions around 28 S (7).

Preparation of cDNA and cloning. cDNA was prepared from the partially purified $mRNA_{vit}$ preparation by reverse transcription (9,10). The synthesis of the respective double stranded DNA and the tailing were performed according to Higuchi et al. (9) and Röwekamp and Firstel (10), respectively. For the incorporation of the DNA into the plasmid pBR322 and for the cloning established methods were applied (10,11).
Clones containing $cDNA_{vit}$ were identified by hybridization with a ^{32}P-labelled cDNA prepared from the partially purified $mRNA_{vit}$ according to Grunstein and Hogness (11).
Plasmid-DNA was obtained from a bacteria clone by the method of Clewell and Helinski (12). The insert ($cDNA_{vit}$) from one of the plasmids containing 1250 bp was used, after labelling with ^{32}P according to Maniatis et al. (13), as a probe for measuring vitellogenin mRNA sequences by "Northern blotting".

Determination of $mRNA_{vit}$. 0.75 µg poly(A)-RNA (see above) from the liver of chicks treated with estradiol or estradiol/tamoxifen was applied to an 0.9% agarose gel and electrophoretically separated (14). The RNA was transfered from the gel to a nitrocellulose filter and hybridized on the filter with heat denatured ^{32}P-labelled $cDNA_{vit}$ (250,000 cpm Cerenkow) (15). The hybridization was performed in buffer A at 37°C for 16 hrs. Then the filter was washed three times with buffer B at room temperature and four times with buffer C at 37°C. RNA-/^{32}P-DNA hybrids were made visible by autoradiography using a Kodak XR-5 film. The bands on the film were scanned using the spectrophotometer KM3 from Zeiss. A linear relationship of the intensity of the bands and of the amount of $mRNA_{vit}$ was observed in the range

of 50 ng to 1 μg poly(A)RNA from the liver of chicks estrogenized for 72 hrs. This RNA (72 hrs after estradiol) which contains a maximal amount of $mRNA_{vit}$ was used as a standard (100 %) and relative intensities were expressed as percent of this standard.

RESULTS

We developed a method which allowes to measure relative amounts of vitellogenin mRNA ($mRNA_{vit}$). Total RNA was extracted from the liver of chicks treated with estradiol or estradiol/tamoxifen. From this preparation RNA was partially purified on poly(U)-Sepharose and separated by agarose gel electrophoresis. After transfer of the RNA from the gel to a nitrocellulose filter, $mRNA_{vit}$ on the filter was made visible by hybridization with ^{32}P-labelled vitellogenin cDNA ($cDNA_{vit}$) and subsequent autoradiography (Fig. 1). The intensity of the $mRNA_{vit}$ bands was measured and relative intensities were expressed as percent of a standard (100 %). Always the same sample of RNA from chicks estrogenized for 72 hrs (maximal value) was used as a standard. Thus differences due to slightly varying conditions of hybridization and autoradiography could be eliminated. No $mRNA_{vit}$ band was visible with RNA from untreated animals (not shown). Various times after estradiol or estradiol/tamoxifen synthesis of vitellogenin in liver pieces was

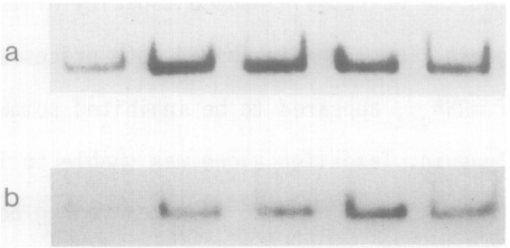

Fig. 1: Northern blotts of various RNA preparations.

a) 24, 48, 72, 96 and 120 hrs after estradiol (25 mg/kg body weight)
b) 24, 48, 72, 96 and 120 hrs after estradiol and tamoxifen (25 mg/kg body weight)

The intensities of the bands in a are not comparable directly with those in b. They have to be expressed as percent of a standard as described in Methods (see Fig. 2).

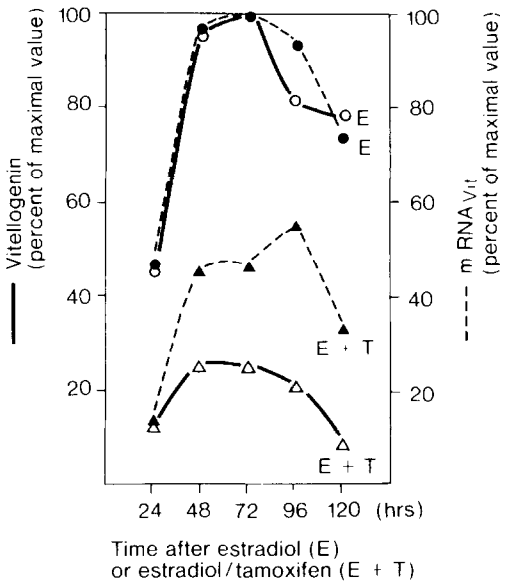

Fig. 2: Vitellogenin and $mRNA_{vit}$ synthesis after estradiol (E) or after estradiol and tamoxifen (E+T)

Relative amounts of $mRNA_{vit}$ were determined as described in Methods (see also Fig. 1). The capacity of chick liver to synthesize vitellogenin was measured in vitro as described previously (5) and is given as percent of the maximal value. At the maximum (100%) vitellogenin synthesis represents 20% of total protein synthesis.

measured and was compared with the relative amounts of $mRNA_{vit}$ extracted from the liver (Fig. 2). Fig. 2 shows that the estradiol-induced production of $mRNA_{vit}$ paralleled the estradiol-induced capacity of the liver to synthesize vitellogenin. Tamoxifen inhibited both processes, even though the accumulation of $mRNA_{vit}$ appeared to be inhibited somewhat less than the synthesis of vitellogenin. Tamoxifen alone was unable to induce the production of $mRNA_{vit}$. Chicks were treated with 25 mg tamoxifen/kg body weight for the times indicated in Fig. 2 as well as with 12.5, 25, 50 and 75 mg tamoxifen/kg body weight for 72 hrs. No $mRNA_{vit}$ band was visible after any of these treatments, even though the amount of RNA (10 ug) after tamoxifen taken for the test was 200-fold larger than that of the RNA (50 ng) 72 hrs after estradiol (not shown). Thus, if any $mRNA_{vit}$ was induced by tamoxifen it was less than 0.5% of that induced by estradiol.

DISCUSSION

To our knowledge the inhibitory effect of antiestrogens has so far not been studied at the level of mRNA production. We could show now that tamoxifen inhibits the estradiol-induced appearance of $mRNA_{vit}$ in chick liver and that this inhibition correlates very well with the reduced capacity of the liver to synthesize vitellogenin. Since total RNA was extracted from liver our data indicate that true inhibition of $mRNA_{vit}$ synthesis and not just inhibition of $mRNA_{vit}$ processing is caused by tamoxifen. We cannot exclude a stimulating effect of tamoxifen on $mRNA_{vit}$ degradation. The latter possibility, however, appears to be rather unlikely. In addition to its inhibitory effect at the transcriptional level, tamoxifen might also affect translation of the $mRNA_{vit}$. An indication for this possibility might be the observation that the inhibition of vitellogenin synthesis is more pronounced than that of $mRNA_{vit}$ synthesis.

In good agreement with previously obtained results on the inability of tamoxifen to induce vitellogenin synthesis (5) we found that $mRNA_{vit}$ is not measurable after tamoxifen alone. From our observation that tamoxifen induces less than 0.5% of the amount of $mRNA_{vit}$ synthesized 72 hrs after estradiol and from the number of 6000 $mRNA_{vit}$ molecules/cell 72 hrs after estradiol as reported by Burns et al. (16), it follows that tamoxifen, if at all, induces less than 30 molecules of $mRNA_{vit}$/cell. Since measuring vitellogenin mRNA sequences with a cDNA probe represents such a sensitive test, it is safe to conclude that tamoxifen indeed lacks any agonistic action in chick liver. Recently we found that tamoxifen (or rather its metabolite monohydroxytamoxifen) is able to translocate the estrogen receptor from the cytoplasm to the nucleus in amounts comparable to those translocated by estradiol (5). The results presented above indicate, however, that this nuclear antiestrogen-receptor complex is unable to induce the synthesis of $mRNA_{vit}$. The reason for this inactivity of the nuclear antiestrogen-receptor complex remains to be elucidated.

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