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# INDUCIBILITY OF THE AVIDIN GENE BY PROGESTERONE IS SUPPRESSED DURING ESTROGEN-INDUCED CYTODIFFERENTIATION

T. JOENSUU,[1]* A. NIEMELÄ,[1] T. KUNNAS,[1] S. SALOMAA,[1] H. ALHO,[1] P. VILJA,[1] T. YLIKOMI,[1] M. KULOMAA[1,2] and P. TUOHIMAA[1]

[1]Department of Biomedical Sciences, University of Tampere, P.O. Box 607, 33101 Tampere and [2]Department of Biology, University of Jyväskylä, 40100 Jyväskylä, Finland

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**Summary**—We have studied epithelial differentiation of the chick oviduct as induced by diethylstilbestrol (DES) and 17$\beta$-estradiol ($E_2$). The proportion of goblet cells in the oviduct was slightly higher after $E_2$ than after DES treatment. Also avidin induction by progesterone was stronger following DES than $E_2$ priming. In the estrogen pretreated oviduct epithelium, avidin expression was induced by progesterone in the surface epithelial cells, protodifferentiated gland cells and tubular gland cells, but not in goblet cells. During prolonged estrogen treatment, however, the inducibility of avidin by progesterone ceased in tubular gland cells but not in surface epithelial cells. The estrogen action on the expression of avidin could be explained by estrogen-induced terminal differentiation of the epithelial gland cells or by a direct effect of estrogen on the progesterone action, for instance interaction of estrogen receptor and progesterone receptor in the regulation of transcription.

## INTRODUCTION

The chick oviduct provides a suitable model system to study growth and differentiation, since differentiation begins late in normal development but can be stimulated by exogenous hormones after hatching. During the posthatching period responses to exogenous hormone treatment are not hampered by endogenous steroid action [1]. Furthermore, the specific responses to progesterone and estrogen in terms of induction of synthesis of avidin and ovalbumin, respectively, have made the system attractive for studies of the mechanisms of steroid hormone action [2–5]. Although estrogen itself does not induce avidin, primary treatment with estrogen is obligatory before progesterone-induced avidin synthesis. The estrogen priming is required for the cytodifferentiation for instance of the goblet cells, which have been thought to be responsible for the production of avidin [3, 6–8]. We have recently shown, however, that avidin is not expressed in the goblet cells, but in the other surface epithelial cells and tubular gland cells [9].

In order to obtain a biological response, considerable concentrations of steroids have to be administered to chicks [10–12]. On the other hand, prolonged experimental treatment with estrogen inhibits the synthesis of avidin in response to progesterone [13]. Diethylstilbestrol (DES), a non-steroidal compound with potent estrogenic activity, has been mostly preferred to natural estrogen during the primary stimulation of the oviduct [3, 9, 14]. However, studies comparing DES and 17$\beta$-estradiol ($E_2$) are lacking. A possible non-physiological response obtained with the administration of exogenous hormone was suggested by Niemelä and Elo who proposed [15] that the differentiation of oviduct epithelium is different following DES and $E_2$ treatments. They showed that in the DES-primed oviducts, but not in the $E_2$-primed one, estrogen administered with progesterone potentiated avidin production [15]. It must also be borne in mind that DES is known to be teratogenic and may act differently from natural estrogen during cellular differentiation [16–19]. We have recently reported that the induction of avidin gene expression in the DES-primed oviduct epithelium can be significantly different compared to that in the oviduct epithelium of chicks and laying hens during physiological development where no exogenous steroid hormones are administered [9].

In the present study we have compared the effects of DES to those of physiological $E_2$ as

*To whom correspondence should be addressed.

inducers of epithelial differentiation. Our purpose was to establish whether the observed differences in avidin expression by progesterone during normal development and DES-induced differentiation, particularly the decrease in avidin expression in glandular cells, are due to inherent non-steroidal effects of DES or to a dose–response difference between DES and $E_2$.

## EXPERIMENTAL

### *Animals*

Newly hatched White Leghorn chicks were obtained from a local chicken farm (Mäkelä, Vilppula). Two to five days after hatching, chicks were injected i.m. with DES obtained from E. Merck (Darmstadt, Germany) or $E_2$ from Sigma (St Louis, MO) (0.05, 0.5 or 5.0 mg/day) for 2, 6, 12 or 18 days. These doses and treatment schedules have been presented in numerous previous works reviewed by Tuohimaa *et al.* [20]. DES and $E_2$ were dissolved in propylenglycol (from Fluka AG, Bucks., England) at different concentrations. The final volume injected was 0.05 ml in all groups. Twenty-four hours after the last estrogen treatment, the chicks were weighed and half of each group received progesterone (from E. Merck) i.m. 20 mg/kg body weight and the rest vehicle (propylenclycol) only. Avidin induction by progesterone has been shown to be dose-dependent at doses of 0.2–20 mg/kg body weight [21]. Twenty-four hours after progesterone injection the chicks were sacrificed and the magnum portion of the oviduct immediately dissected, weighed, frozen in liquid nitrogen and stored at −80°C until assay. Part of the oviduct tissue was cut and fixed in Baker's fluid (4% paraformaldehyde, 1% $CaCl_2$) at 4°C for 2 h, dehydrated and embedded in paraffin.

### *IEMA for avidin and ovalbumin*

The concentration of avidin and ovalbumin was measured by an immunoenzymometric assay (IEMA) as described previously [21]. Briefly, standards or specimens were incubated at 37°C for 1 h on a microtitre plate (Maxisorb, Nunc, Roskilde, Denmark) precoated with avidin or ovalbumin antibody. Anti-avidin or -ovalbumin peroxidase conjugate was then added and incubated at 37°C for 1 h. The colour reaction was visualized with an enzyme substrate (*o*-phenylenediamine) and absorbances measured at 492 nm. The detection limit in both IEMAs was 0.1 ng/ml and the determination range 0.5–100 ng/ml.

### *Detection of estrogen receptor (ER)*

Immunohistochemical analysis of ER was performed as described previously [22] using the ER-ICA kit (Abbott Labs, North Chicago, IL). The monoclonal ER antibody H222 has been shown to recognize chicken ER [22, 23].

### *RNA isolation and transfer*

Frozen oviducts were pulverized immediately prior to homogenization and total RNA was isolated using acid guanidium thiocyanate–phenol–chloroform extraction [24]. For RNA hybridization analysis, 10 $\mu$g of total RNA was separated by size on 1.5% formaldehyde–agarose gel and transferred to a nitrocellulose filter (Hybond C, 0.45 $\mu$m; Amersham Int., Bucks., England) by capillary blotting [25]. Hybridization analysis was carried out as described previously [25a]. RNA ladders from BRL (Gaithersburg, MD) were used for size standards. RNA was fixed to the filter by baking at +65°C overnight.

### *Preparation of cDNA probes*

The inserts of the recombinant DNA clones for avidin [5] and ovalbumin [26] were digested by restriction enzymes to obtain fragments of about 400–600 bp in length. The plasmid pBR322 digested with HinfI was used as a negative control. For *in situ* hybridization, the fragments were labelled by random priming with digoxigenin dUTP according to the instructions provided by the manufacturer of the kit (Genius, Boehringer Mannheim). Radioactive probes were prepared by nick-translation to a sp. act. of $1–4 \times 10^8$ or $1.6 \times 10^8$ cpm/$\mu$g by [$^{32}$P]dCTP or [$^{35}$S]dATP (Amersham), respectively.

### *In situ hybridization*

Thin paraffin sections (4 $\mu$m) were mounted on subbed slides (poly-L-lysine, 0.05%), deparaffinized with xylene and rehydrated in graded ethanol. Prior to hybridization the sections were treated with proteinase-K (5 $\mu$g/ml, in 10 mM Tris–Cl, 2 mM $CaCl_2$, pH 7.5) for 5 min at 37°C. After washing (2 × 5 min with 2 × SSC), the sections were incubated with prehybridization buffer (0.6 M NaCl, 10 mM Tris, 1 × Denhardt's, 500 $\mu$g/ml salmon sperm DNA and 50 $\mu$g/ml yeast tRNA and 50% formamide) at 37°C for 1 h. The hybridization mixture

contained 10 pg/μl and 30 pg/μl $^{35}$S- and digoxigenin-labelled probe, respectively, and 10% (wt/vol) dextran sulphate added to hybridization buffer; the $^{35}$S-labelled probe mixture also contained 10 mM dithiothreitol (DTT). The prehybridization buffer was removed and the heat-denatured hybridization buffer applied to the sections covered with coverslips and hydridized at 42°C for 24 h. After hybridization the coverslips were removed and the sections washed with 2 × SSC for 2 × 10 min, 1 × SSC for 1 h and 0.5 SSC for 2 h at RT (20–25°C). The digoxigenin-labelled hybrids were detected with the Genius kit according to the manufacturers' instructions (Boehringer Mannheim). Specimens hybridized with $^{35}$S-labelled probes were, after washing, dehydrated through graded alcohols, dried, dipped in photographic emulsion (nuclear Track NTB-2 emulsion, Eastmann Kodak) and exposed in dark boxes at 4°C for 5–7 days.

*Histology*

For histological study some sections were prepared and analysed as described previously [9]. Briefly, the general structure was visualized by hematoxylin-eosin (HE) staining and mucus-producing goblet cells were specifically stained with alcian blue. The goblet cells were counted from the surface epithelium in microscopic view of 250× magnification, both after 18 days DES and $E_2$ treatments, when the difference in progesterone-induced avidin production following estrogen and DES treatments disappeared and the oviduct was fully differentiated.

## RESULTS

*Avidin IEMA and avidin mRNA*

The avidin induction by progesterone after different estrogen treatments is shown in Fig. 1. The dose of progesterone was kept constant and chosen to produce the maximal response in the expression of avidin, since we were interested in the effects of different estrogen treatments on avidin induction by progesterone. Avidin was induced only by progesterone, not by estrogen alone even at the highest dose (5 mg) for 18 days. Statistically significant ($P < 0.05$, Student's $t$-test) induction of avidin by progesterone (20 mg/kg i.m.) was already observed after 2 days of estrogen treatment at a dose of 5 mg of DES or $E_2$, and after 6 days at a dose of 0.05 mg of DES or 0.5 mg of $E_2$ (Fig. 1). Maximal stimulation of avidin was observed after DES (0.05 mg) and $E_2$ (5 mg) for 12 and 6 days, respectively. Maximal stimulation was higher after DES than $E_2$ pretreatment. A significant decrease in stimulation of avidin by progesterone was seen during prolonged estrogen treatment. The decrease was observed between 6 and 12 days for 5 mg of $E_2$ ($P$-value 0.003) and between 12 and 18 days for DES ($P$-values for doses of 0.05, 0.5 and 5 mg being 0.006, 0.001 and 0.03, respectively). After 18 days' treatment there was no difference ($P$-values $>0.05$) in avidin expression between different doses and DES and $E_2$ treatments.

We then sought to ascertain whether the decrease in avidin content in the oviduct after prolonged $E_2$ treatment could be observed at mRNA level. On the basis of changes at the protein level described in Fig. 1 we were especially interested in the 12 days' treatment. At this time the maximal avidin stimulation had already taken place with the high dose of $E_2$. Figure 2 shows RNA hybridization analysis of the total oviduct RNA with the avidin-specific probe. After estrogen pretreatment for 12 days, avidin mRNA induction by progesterone was highest after a daily dose of 0.05 mg of $E_2$ and lowest after 5 mg of $E_2$.

*Avidin in situ hybridization*

An *in situ* hybridization technique was used to determine avidin-producing cell types during estrogen-induced oviduct differentiation. The sensitivity and specificity of digoxigenin-labelled cDNA probes were compared with those of $^{35}$S-labelled probes. The location of mRNAs and the sensitivity of hybridization signals were similar with digoxigenin or radioactive probes, as shown previously [27]. The digoxigenin-labelled probes were chosen by reason of their non-radioactivity, rapidity and easy use compared to $^{35}$S-labelled probes. After a short (1 day with 5 mg of) $E_2$ or DES treatment progesterone induced avidin expression in some cells in the surface epithelium (Fig. 3, top left). At this stage the epithelium is beginning to fold and these invaginating cells in the surface epithelium, named protodifferentiated tubular gland cells by Palmiter and Wrenn [28], express avidin mRNA. These cells further differentiate into proper tubular gland cells. After a 6 days' treatment the tubular glands were formed. Figure 3, top right, shows that at this stage avidin expression in some gland cells has

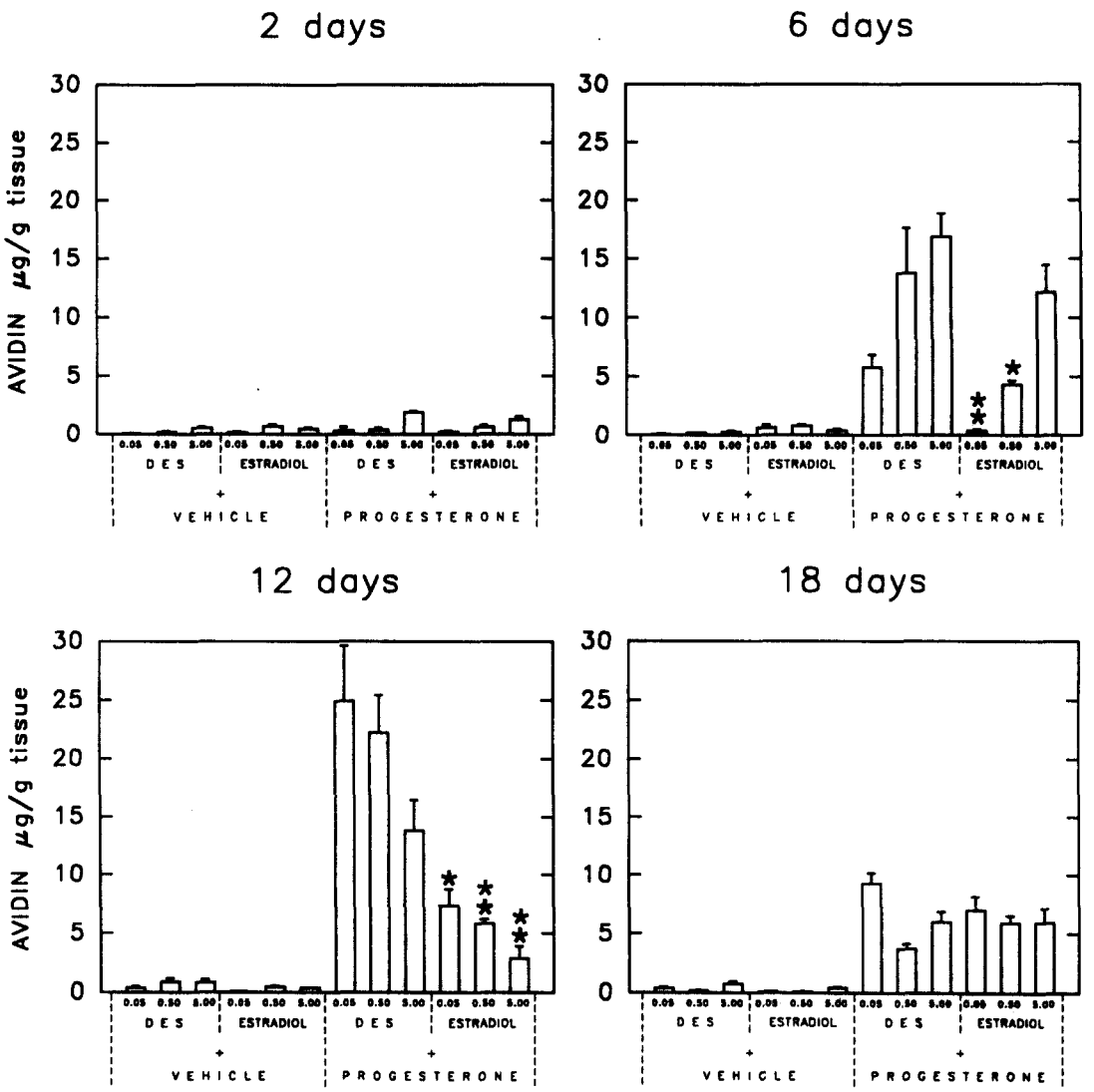


Fig. 1. Effect of dose and duration of estrogen pretreatment on avidin production by progesterone detected by IEMA. Avidin production after different times (2, 6, 12 or 18 days) of estrogen treatment (DES or $E_2$ 0.05, 0.5 or 5 mg/day) following a single dose of progesterone (20 mg/kg i.m. for 24 h *in vivo*) is shown in separate panels: 2 days, top left; 6 days, top right; 12 days, bottom left; 18 days, bottom right. Each bar represents the amount of avidin (mean ± SEM) in the oviduct magnum mucosa of 5–8 samples in groups. **P*-value <0.05 and ***P*-value <0.005, compared with the corresponding dose of DES and $E_2$.

already started to decrease. Avidin was expressed at 6 days' in surface epithelial cells as at the onset of differentiation (1 day). The cells of tubular glands did not express avidin mRNA evenly (Fig. 3, top right): there were cells with different hybridization intensities and also cells with no detectable signal. As a result of prolonged estrogen treatment (12 days), avidin mRNA was synthesized mainly in the surface epithelial cells, and no avidin-expressing cells were seen in the glands. In Fig. 3, bottom left, the cessation of progesterone-induced avidin synthesis in tubular glands, but not in surface epithelial cells, is shown following 12 days' $E_2$-treatment at a dose of 5 mg/day. No hybridization signal was observed with labelled pBR322 used as a negative control (Fig. 3, bottom right).

*Histology*

To assess the potential pathological changes and toxic effects caused by high doses of estrogen, samples for histological analysis were taken from 20 animals receiving 5 mg of DES or $E_2$ for 12 or 18 days. None of the specimens contained tumours or abnormal mitose patterns, but the basal lamina was continuous beneath the epithelium (Fig. 4). Granular eosinophilic staining in tubular glands reflected active protein

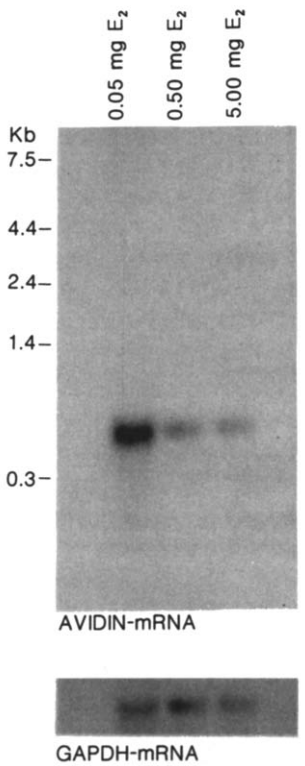


Fig. 2. Effect of $E_2$ pretreatment on avidin mRNA induction by progesterone. The chicks were pretreated with different amounts of $E_2$ for 12 days and thereafter received a single injection of progesterone (20 mg/kg i.m.). Total RNA was isolated and RNAs separated in denaturing conditions. After the transfer, the filter was hybridized using a $^{32}$P-labelled cDNAs for avidin and GAPDH as probes.

synthesis induced by estrogen. After 18 days of treatment, goblet cells were the main surface epithelial cell type observed (Fig. 4, top and bottom left). The proportion of goblet cells in the epithelium was higher (about 50%, *P*-value $<0.05$) after $E_2$ than after DES treatment. High doses of estrogen ($E_2$ or DES) also induced development of an oviduct on the right side of the abdominal wall. These "pseudo-oviducts" occasionally contained cysts filled with fluid in the serosal layer, but the glandular structure in the epithelium was histologically indistinguishable from the "real" oviduct.

### *Ovalbumin in situ hybridization, IEMA and ER*

The decrease in the progesterone-induced avidin gene expression appeared to be dependent on estrogen pretreatment and coincided with the cessation of avidin synthesis in the tubular gland cells. Therefore, we studied by an *in situ* hybridization technique the expression of estrogen-dependent ovalbumin mRNA and ER at the stage when the expression of avidin in the tubular glands had ceased. After 5 mg of $E_2$/day for 18 days the ER immunoreactivity was strong in all tubular gland cells, whereas faint staining was seen in some surface epithelial cells (Fig. 4, top right). The lower expression of ER was also in good agreement with the much lower expression of ovalbumin mRNA in the surface epithelial cells compared to the tubular gland cells (Fig. 4, top and bottom right). In order to make a comparison between avidin and ovalbumin gene expression we also measured the amount of ovalbumin (Table 1) following maximal hormone stimulation (5 mg of DES or

*(Figs 3 and 4 overleaf)*

Fig. 3. *In situ* hybridization of avidin in the chick oviduct with digoxigenin-labelled 550 bp fragments of avidin cDNA probe. The time of estrogen pretreatment (5 mg of $E_2$/day) was 1, 6 or 12 days (shown in the figure), after which chicks received a single injection of progesterone (20 mg/kg, 24 h *in vivo*). Top left: after the *onset of differentiation* the epithelial cells budding to the stroma synthesize avidin. Top right: after the formation of tubular glands in the *differentiating oviduct*, avidin is synthesized in both the surface epithelium and tubular gland cells, but at this stage there is heterogeneity between adjacent gland cells in the expression of avidin. Bottom left: following high doses of estrogen for 12 days, avidin is expressed in the *differentiated oviduct* mainly in the surface epithelium. Bottom right: in the *in situ* control study (5 mg of $E_2$/day for 6 days plus progesterone) 550 bp HinfI fragments of pPR 322 was used instead of avidin mRNA specific probe. Arrowheads show avidin-expressing surface epithelial and protodifferentiated gland cells. Bold arrows indicate avidin-expressing tubular gland cells. Open arrows indicate avidin-negative tubular gland cells. L, lumen of the oviduct; E, surface epithelium; S, stroma; TG, tubular glands. Bar = 25 $\mu$m. No counterstain.

Fig. 4. Location of ER and *in situ* hybridization of ovalbumin. HE staining of the chick oviduct epithelium after 18 days' treatment with 5 mg of estrogen/day is shown for DES in the top left and for $E_2$ in the bottom left; note the normal histological structure and the main surface epithelial cell type (goblet cell), which are more numerous following $E_2$ than DES treatment. Top right: immunoreactivity of ER after 18 days' $E_2$ treatment (5 mg/day) with monoclonal antibody H222 is located in the gland cells (arrowheads); some faintly positive cells also in the surface epithelium (shown by arrows). In the immunohistochemical control study normal rat IgG was used instead of primary antibody. Bottom right: *in situ* hybridization of ovalbumin in the chick oviduct following estrogen (5 mg of $E_2$/day for 12 days) plus progesterone treatment (same sample as in Fig. 3 for avidin *in situ* at 12 days' treatment). Reaction is mainly in the tubular glands. Faint reaction is shown in a few surface epithelial cells (bold arrows). The hybridization control is shown in Fig. 3, bottom right. E, surface epithelium of the chick oviduct magnum mucosa; G, tubular glands. Bar = 20 $\mu$m. No counterstain.

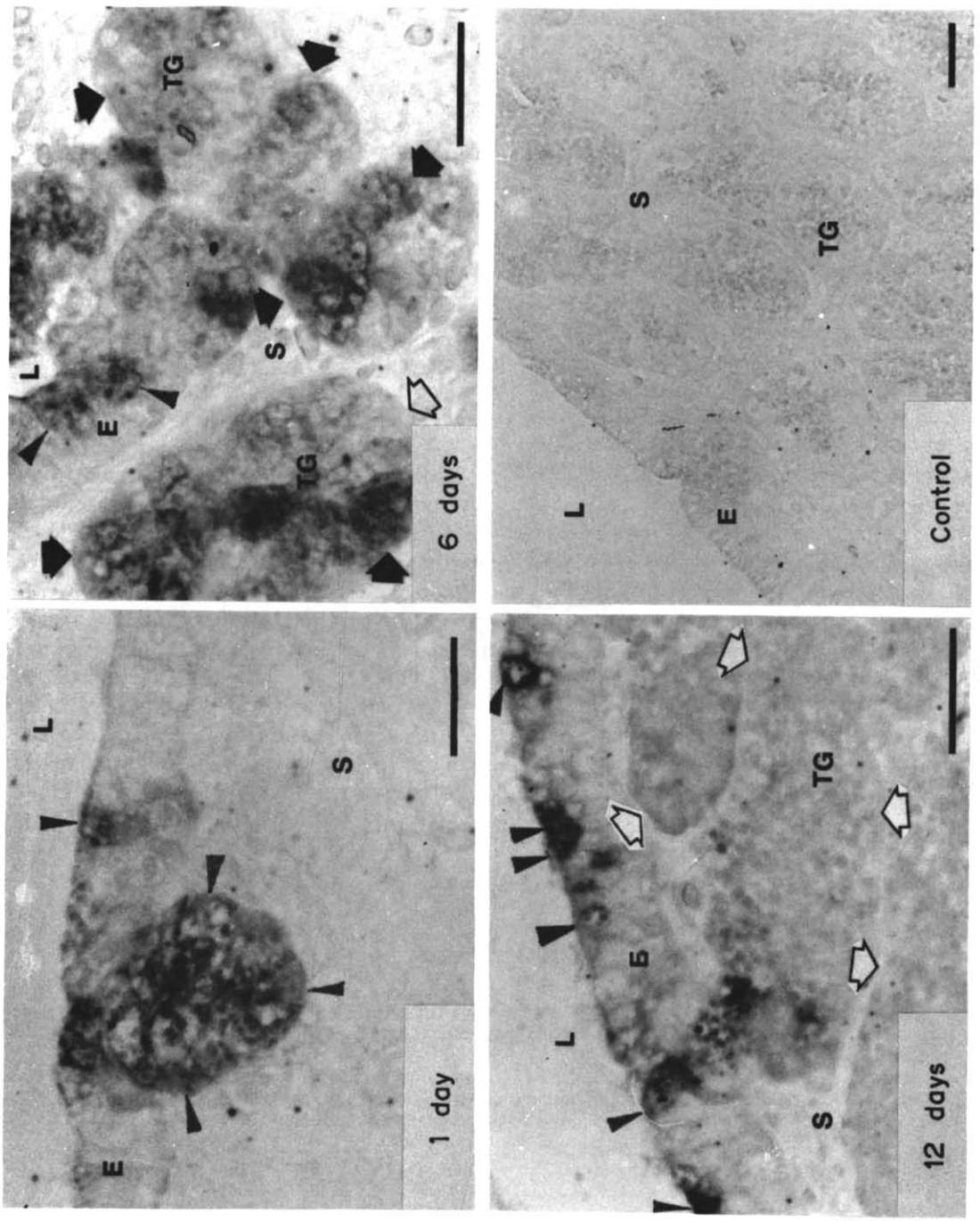


Fig. 3—*legend on p. 613.*

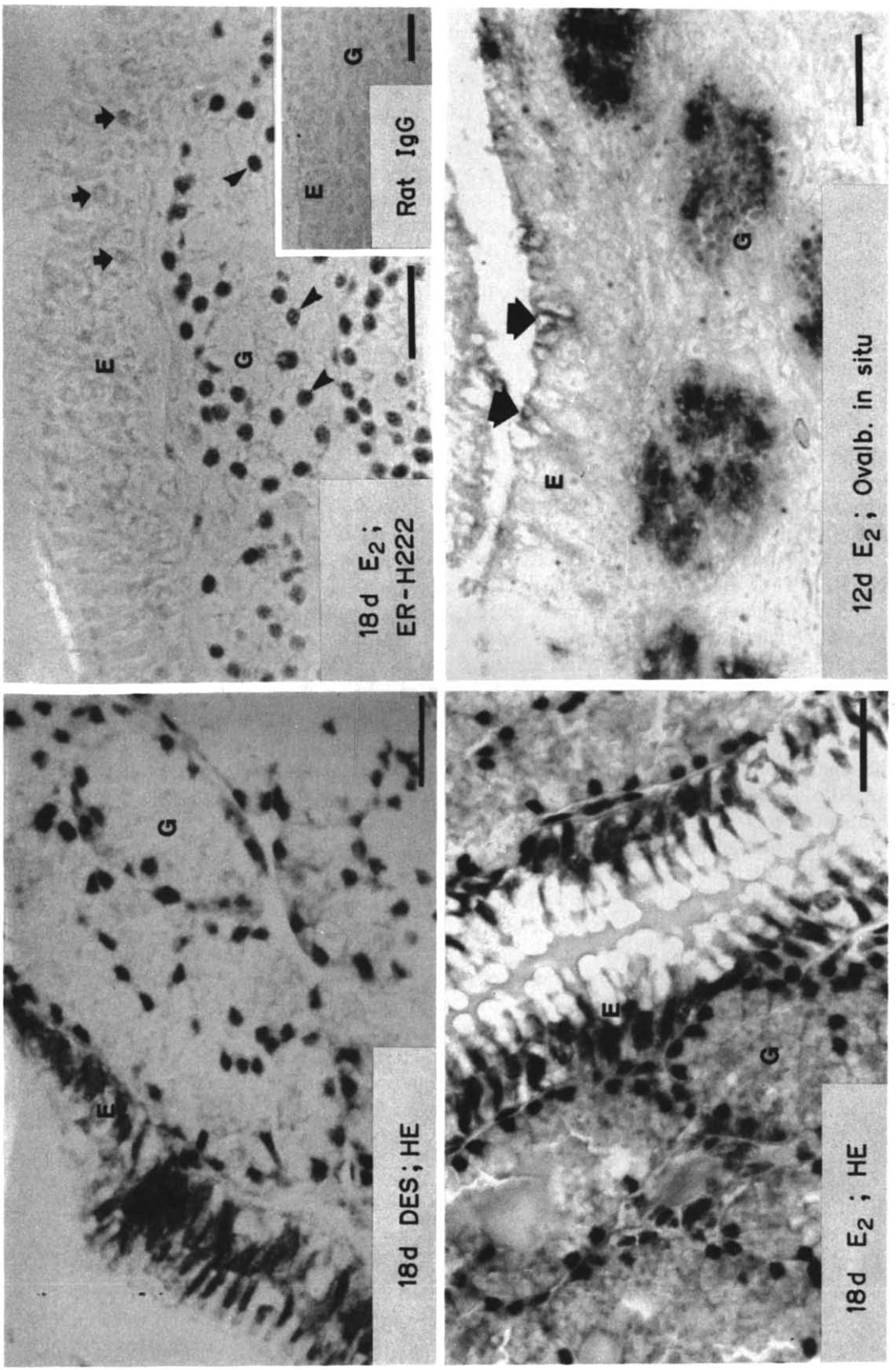


Fig. 4—*legend on p. 613.*

Table 1. The production of ovalbumin [mg/g tissue + SEM ($n$)] after high doses (5 mg/day) of estrogen plus a single injection of progesterone (20 mg/kg) detected by ovalbumin IEMA

| Time of treatment | DES | $E_2$ |
|---|---|---|
| 6 days | 65.3 + 3.5 (6) | 35.0 + 2.4 (6) |
| 12 days | 113.0 + 15.4 (7) | 110.0 + 10.5 (6) |
| 18 days | 114.6 + 14.0 (5) | 102.5 + 11.9 (5) |

The difference between 6 and 12 days was significant ($P$-value $<0.05$). The differences between 12 and 18 days' treatments were non-significant ($P$-value $>0.05$).

$E_2$/day for 6, 12 and 18 days followed by progesterone 20 mg/kg for 24 h *in vivo*). The maximal level of ovalbumin expression was achieved following 12 days' treatment and the quantity of ovalbumin production also remained on that level following 18 days' treatment.

## DISCUSSION

The teratogenic and carcinogenic effects of DES [16–19] must be considered when DES-induced differentiation [9, 15] is compared with normal development. We have observed, however, no marked differences between responses to DES and $E_2$. Furthermore, it is likely that $E_2$ is also carcinogenic if used over long periods of time or at high doses [29]; the effect depends primarily on the estrogenic potency, characterized by different affinity for the receptor and different pharmacokinetics and metabolisms of DES and $E_2$ and the time of estrogenic influence [30–32]. In the histological samples no tumours or aberrant mitoses were found, but as a developmental disorder both DES and $E_2$ caused differentiation of the oviduct on the right side of the abdominal wall. This oviduct normally regresses possibly due to the action of androgens [33]. The atrophic effect, however, appeared to be overridden by high doses of estrogen.

The induction of avidin by progesterone was higher at the beginning of differentiation following DES than $E_2$. This could reflect differences in the mechanism of action of these two compounds. Our interpretation of the difference is that it reflects rather different differentiation stages after DES and $E_2$ priming and different estrogenic potencies. Since avidin is a good marker of cytodifferentiation [3, 7], it appears for example that following 6 days' treatment with 0.05 mg of DES the epithelial differentiation had started, but after 6 days' treatment with 0.05 mg of $E_2$ the epithelium was still non-differentiated. This was confirmed from the histological samples. The higher avidin induction after DES than $E_2$ priming could be explained in part by the observed slightly altered representation of different cell populations induced by DES and $E_2$ (more goblet cells after $E_2$ priming), which is in good agreement with the finding that avidin is not produced by goblet cells [9, 34]. This does not, however, totally explain the difference, since the differences in avidin production disappeared after full differentiation, although there appeared to be a difference in the ratio of goblet/surface epithelial cells.

The ability of cells to express avidin appeared to depend on their differentiation stage. Progesterone did not induce avidin in the non-differentiated oviduct, in agreement with previous studies [3, 9, 28, 35]. Thus the estrogen-induced cytodifferentiation of the oviduct epithelium is a prerequisite for the cells to produce avidin following progesterone treatment. During the first days of estrogen administration, a potentiation of the progesterone-induced avidin synthesis was seen. Progesterone had a maximal effect on avidin gene expression after 6 or 12 days (depending on the dose) of estrogen treatment, whereafter, the avidin response to progesterone decreased significantly. The decrease in avidin induction by progesterone was seen not only following DES but also after $E_2$ pretreatment. This appeared to be due to the cessation of avidin synthesis in tubular gland cells as suggested for DES in a previous study based on immunohistochemical analysis using an affinity-purified polyclonal antibody [9]. The changes in avidin expression and location can be explained by different estrogen pretreatment schedules and by a different differentiation status of the epithelium. In addition to the surface epithelial cells, estrogen caused the onset of cytodifferentiation in protodifferentiated tubular gland cells capable of synthesizing avidin. These cells had started the invagination into the underlying stroma at the very beginning of differentiation. It is essential that avidin was found in glandular cells only during early stages of differentiation; shortly after the formation of tubular glands. But not in the differentiated glands after long estrogen treatment. A specific mechanism for the suppression and regulation of avidin gene expression is suggested by the fact that the production of ovalbumin did not decrease after long estrogen pretreatment with high doses followed by a single treatment with progesterone. Since ovalbumin, the major protein in egg white albumen [36], is also a marker

of cytodifferentiation [28, 37–40] the unchangeable production of ovalbumin after 12 days' treatment with maximal hormone stimulus indicates that the oviduct epithelium was fully differentiated after 12 days' treatment. This is also supported by the fact that the amount of ovalbumin per gram of tissue was on the same level or slightly higher to that which we had detected in the oviduct of the laying hen [19].

The mechanism of estrogen action may be explained by estrogen-induced terminal differentiation of the epithelial gland cells. The tubular gland cells lose their capacity to express the avidin gene during terminal differentiation of the cells, unlike surface epithelial cells. It seems plausible that high doses of estrogen induce a rapid differentiation of the oviduct, whereas with low doses of estrogen cytodifferentiation is a slow process. It is known that progesterone does not induce avidin in the oviduct of aged non-laying hens [41]. However, in a previous study we have shown that avidin gene expression in the tubular gland cells reappeared after withdrawal of exogenous estrogen (DES) [9]. This suggests that estrogen is needed for the maintenance of differentiated phenotypes of the cells. The action of estrogen on the tubular gland cells was supported by the shown distribution of ER and estrogen-regulated expression of ovalbumin mRNA. In the differentiated oviduct the tubular gland cells were shown to be without doubt the target cells for estrogen: they contained ER and expressed ovalbumin following estrogen treatment. It was conspicuous that following high doses of estrogen avidin was expressed by progesterone in the ER-negative, but not in the ER-positive cells.

It has been shown that gene expression by the progesterone receptor (PR) can be inhibited by ER in a dose-dependent manner by a mechanism called transcriptional interference [42]. We have shown that virtually all of the tubular gland cells express both ER and PR [9, 22, 43]. Thus the prolonged and/or high doses of estrogen treatment might inhibit progesterone functions, like avidin induction, by the same mechanism. However, as yet we have no reliable methods for the quantitation of ER and PR inside cell nuclei in histological samples, although it clearly appeared that the immunoreactivity of ER was negligible in surface epithelial cells when compared to the tubular gland cells. Furthermore, this cannot totally explain the observed cessation of avidin expression in the gland cells during estrogen treatment, since it is a rather long process (1–2 weeks). And also because between days 6–12 there are gland cells, which contain both receptors [9, 22, 43] and still express avidin. Thus it is probably that the suppression of avidin gene expression during estrogen treatment is caused by cell differentiation, but it cannot be ruled out that it is partially due to transcriptional interference of progesterone action by ER.

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