A synthetic oestrogen antagonist, tamoxifen, inhibits oestrogen-induced transcriptional, but not post-transcriptional, regulation of gene expression

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Oestrogen (E2) regulates the expression of its target genes at transcriptional and post-transcriptional levels. To clarify the mechanism of E2-induced post-transcriptional regulation, with attention to the involvement of the oestrogen receptor (ER), we studied the effect of tamoxifen (TAM), a synthetic E2 antagonist that inhibits ER-mediated transcription, on E2-induced transcriptional and post-transcriptional regulation of the chicken ovalbumin (OVA) gene in chick oviducts. Run-on analysis with oviduct nuclei isolated from E2-treated chicks showed that TAM treatment completely blocked E2-induced transcription of the OVA gene within 24 h without affecting ER gene expression. Likewise, the rate of transcription fell to below the limit of detection after E2 withdrawal from the chicks. Reflecting the

transcription rate, OVA mRNA accumulated linearly in E2-treated chicks, and E2 withdrawal caused a rapid loss of OVA mRNA. However, in the chicks treated with TAM and E2, OVA mRNA was degraded slowly over 48 h with a half-life of 24 h, suggesting that TAM does not inhibit E2-induced mRNA stabilization. Moreover, E2-induced mRNA stabilization was observed even when transcription of the OVA gene was blocked by a transcription inhibitor. Western-blot analysis showed that the remaining OVA mRNA was translatable. Thus the present study indicates that E2 regulates expression of the OVA gene via distinct pathways at transcriptional and post-transcriptional levels.

INTRODUCTION

Oestrogen (E2) regulates expression of its target genes at both transcriptional and post-transcriptional levels [1]. It is generally accepted that transcriptional control of the target genes by E2 is mediated via a nuclear oestrogen receptor (ER), which is a member of the nuclear receptor superfamily and acts as a hormone-inducible transcription factor [2–4]. ER binds as a homodimer to E2-response elements in target gene promoters and consequently controls transcription [5–7]. However, little information is available on the molecular mechanism by which E2 regulates post-transcriptional events, i.e. E2-induced stabilization of mRNAs [8–10], although it has been shown that the half-lives of mRNAs are controlled by cytosolic factors bound to their untranslated regions [1,11,12]. The involvement of ER in E2-induced post-transcriptional regulation remains unclear [13,14]. Tamoxifen (TAM), a non-steroid E2 antagonist, is known to block E2-induced transcription by binding to ER [15]. TAM has therefore been used in endocrine therapy for breast cancer patients with ER positive tumours, although it shows a partial agonistic activity in some patients [16].

These observations led us to investigate whether a compound that antagonizes E2-induced transcription inhibits E2-induced post-transcription. In the present study, we examined the effects of TAM on the E2-induced transcriptional and post-transcriptional regulation of chicken ovalbumin (OVA) gene expression in chick oviducts. It is well known that E2 strictly regulates the expression of this gene in avian oviducts [17–19]. The present report shows that, although TAM clearly suppressed the E2-induced transcription of the OVA gene without affecting

ER gene expression, it did not block the E2-induced post-transcriptional control (OVA mRNA stabilization), and the remaining mRNA appeared to be able to be translated to protein. These findings imply that the E2-induced mRNA stabilization is independent of the ER-mediated transcriptional events in OVA gene expression.

MATERIALS AND METHODS

Animals

E2 was administered to 3-day-old Dekalb white leghorn chicks by subcutaneous implantation of a silicone tube (Dow Corning Co.) containing 35 mg of a synthetic E2, diethylstilboestrol (DES), for 14 days (primary stimulation). Withdrawal was achieved by removing the tube for 5 days [10]. Secondary stimulation was performed by intramuscular injection of either DES (2 mg/chick) or TAM (10 mg/chick) or both dissolved in propyleneglycol [20], and chicks designated as withdrawal were given vehicle alone. To block transcription of the OVA gene, actinomycin D (0.4 mg/chick) was introduced by intramuscular injection 1 h before secondary stimulation [21]. The chicks were killed at the times indicated in the Figures and the magnum portion of the oviduct was excised for analysis.

RNA extraction and quantification of mRNA

Total RNA was isolated from the oviducts by the acid guanidinium thiocyanate/phenol/chloroform method [22]. Total

Figure 1 Effect of TAM on E2-induced transcription of the chicken OVA gene

Chicks were pretreated with DES (2 mg/chick) for 24 h. At time 0, the pretreated chicks were divided into four groups and each group of five birds was treated with either 2 mg of DES/chick (DES), 10 mg of TAM/chick (WD + TAM) or both (DES + TAM) or treated with vehicle alone (WD) every 24 h. The chicks were killed at the times indicated, and oviduct nuclei were isolated and a nuclear run-on assay was performed as described in the Materials and methods section. pBR322 (parent vector) was used as a negative control. (a) Representative results of the nuclear run-on assay are shown. (b) The relative transcription rates of the OVA gene were normalized against the transcription rates of the calmodulin gene and are expressed as means $\pm$ S.D. of more than five samples from different chicks.

RNA (2 or 20 μ g) was fractionated on a 1.1 M formaldehyde/1% agarose gel, then transferred to a nylon membrane (Gene Screen; NEN) by capillary blotting as described previously [23]. To estimate the levels of OVA mRNA, total RNA (2 μ g or 20 μ g) for each sample was blotted on to a nylon membrane with a slot-blotting apparatus [10]. The blots were hybridized overnight with 32 P-labelled cDNA probes (OVA gene cDNA [24] and calmodulin cDNA [25]) as described previously [10]. To obtain quantitative data for OVA mRNA (more than five samples), densitometric analysis of the autoradiograms of the transcripts was performed and the calmodulin transcript was used as an internal control for normalization of the samples [10].

Nuclear run-on transcription assay

Isolation of nuclei and the run-on transcription assay were performed as described previously [26]. Frozen oviducts were homogenized and centrifuged at 90000 g at 4 $^{\circ}$ C for 90 min, and the nuclei were stored at -80° C until analysis. Isolated nuclei including 30 μ g of DNA were incubated at 32 $^{\circ}$ C for 30 min in reaction mixture containing 25 μ Ci of [α - 32 P]CTP (650 Ci/mmol; ICN). The reaction was terminated by the addition of DNase I and proteinase K. RNA was extracted with phenol/chloroform (1:1, v/v) and precipitated with trichloroacetic acid. Radio-labelled RNA (5×10^6 c.p.m.) was hybridized to cDNA plasmids (OVA cDNA [27] and calmodulin cDNA [25]), and the filters were exposed to X-ray film at -80° C. The relative rate of

OVA transcription was measured by densitometric analysis of the autoradiograms and is shown after normalization against the level of calmodulin transcript [10].

Reverse transcription PCR

Specific chicken ER and β -actin cDNAs were generated with pairs of oligonucleotide primers specific for chicken ER (nucleotides 1131–1150 and 1296–1315 of the E domain in chicken ER gene) and β -actin (nucleotides 2029–2048 of exon 4 and 2509–2528 of exon 5 in β -actin gene). Specific cDNAs were amplified by PCR. One cycle of PCR consisted of 94 $^{\circ}$ C for 50 s, 58 $^{\circ}$ C for 70 s and 72 $^{\circ}$ C for 120 s, and was repeated 24 times for chicken ER and 19 times for β -actin [28]. PCR products were electrophoresed in 1% agarose gels and transferred to nylon membranes. Hybridization was performed as described previously [28] and the full-length cDNAs of chicken ER [29] or mouse β -actin [30] were used as hybridization probes. The relative level of chicken ER was measured by densitometric analysis of the autoradiograms and is shown after normalization against β -actin transcript [28].

Western-blot analysis

Tissue was homogenized in a buffer containing 0.1 M Tris/HCl, pH 7.5, and 0.1 M 2-mercaptoethanol, and the homogenate was centrifuged at 27000 g for 20 min. The supernatants were

separated by electrophoresis on SDS/12.5% polyacrylamide gels and transferred to nitrocellulose membranes in buffer containing 125 mM Tris, 960 mM glycine and 20% methanol, pH 9.0. After blocking with PBS containing 1% dried milk, OVA protein was detected with an OVA-specific polyclonal antibody [31] using peroxidase-conjugated anti-rabbit IgG (Organon Teknika Co.). The blots were washed in PBS containing 0.1% Tween 20, and developed using an immunostaining kit (Wako Chemical).

RESULTS

Effect of TAM on the transcriptional and post-transcriptional regulation of the chicken OVA gene in E2-treated chick oviducts

It is well known that E2 strictly controls expression of the OVA gene in chick oviducts at both transcriptional and post-transcriptional levels [17–19]. Although the molecular mechanism of the transcriptional control has been investigated intensively in studies of the OVA gene promoter [7,32,33], there is little information on the molecular mechanism by which E2 stabilizes OVA mRNA [8,10]. In particular, the involvement of ER in E2-induced mRNA stabilization remains unclear [13,14]. To address these issues, the effects of TAM, a synthetic E2 antagonist, on the E2-induced stabilization of OVA mRNA and transcription of the OVA gene were estimated by means of slot-blotting and *in vitro* run-on analysis. Chicks were treated with a synthetic E2,

DES, for 24 h and then divided into four groups (time zero in the Figures). Three groups were treated again at 24 h with DES (DES in Figures), TAM (WD+TAM in Figures) or both (DES+TAM in Figures). Chicks in the withdrawal groups were treated with vehicle alone (WD in Figures).

Transcription of the OVA gene was induced by E2 treatment, and the relative rate of transcription was constant over 48 h (DES in Figure 1). Withdrawal of E2 caused a decrease in transcription, and the rate became negligible within 24 h (WD in Figure 1). As expected from previous findings [20,34], TAM completely suppressed the transcription of the OVA gene induced by E2 (DES+TAM in Figure 1).

When the chick oviducts were continuously treated with E2, OVA mRNA was linearly accumulated (DES in Figure 2). In chicks in which E2 was withdrawn, OVA mRNA disappeared quickly as a result of retarded transcription (WD in Figure 2) and destabilization of the OVA mRNA, as the absence of E2 is known to shorten the half-life of OVA mRNA (from 24 to 4 h) [10]. Indeed, taken together with the preliminary results, the half-life of OVA mRNA in the E2-withdrawal group was estimated to be 4–6 h from the decay of the mRNA (Figure 2). Although the presence of TAM, like E2 withdrawal, completely abolished transcription of the OVA gene induced by E2 (DES+TAM in Figure 1), to our surprise some OVA mRNA remained even after 48 h, in sharp contrast with the complete lack of OVA mRNA in the E2-withdrawal group after 24 h (DES+TAM in Figure 2). As transcription was blocked by TAM even in the presence of

Figure 2 Effect of TAM on E2-induced stabilization of OVA mRNA

Chicks were treated as described in the legend to Figure 1. They were killed at the times indicated, and total RNA was extracted from the magnum portion of the oviducts as described in the Materials and methods section. Then 2 μ g (DES) or 20 μ g (DES+TAM, WD and WD+TAM) of total RNA was used for Northern-blot or slot-blot analysis. (a) Representative results of Northern-blot analysis are shown. (b) The relative levels of OVA mRNA were quantified by slot-blot hybridization. The results are shown after normalization against the calmodulin mRNA and are expressed as means $\pm$ S.D. of more than five samples from different chicks.

Figure 3 Independence of E2-induced OVA mRNA stabilization from the transcriptional event

Chicks were pretreated with DES for 24 h as described in the legend to Figure 1, and then injected with actinomycin D (0.4 mg/chick). After 1 h, the chicks were divided into four groups as described in the legend to Figure 1. (a) Relative transcription rates of the OVA gene were calculated and expressed as described in the legend to Figure 1. (b) Relative levels of OVA mRNA were quantified and expressed as described in the legend to Figure 2.

E2, the remaining mRNA suggests a half-life of around 24 h. These results indicate that TAM does not block E2-induced mRNA stabilization.

To clarify this issue, OVA mRNA decay was estimated when transcription of the OVA gene was blocked by a transcription inhibitor (actinomycin D). Actinomycin D treatment caused rapid cessation of transcription (Figure 3a). However, irrespective of TAM treatment, E2-induced mRNA stabilization was not affected by actinomycin D treatment (Figure 3), clearly suggesting that E2-induced mRNA stabilization is independent of ER-mediated transcription of the OVA gene in the chick oviduct. In sharp contrast, rapid degradation of OVA mRNA was observed in the E2 withdrawal groups (Figure 3b).

To determine whether the OVA mRNA remaining in the oviducts of the chicks treated with TAM is translatable, we

Figure 4 Analysis of OVA protein levels in chick oviducts

Chicks were treated as described in the legend to Figure 1. Cytosolic extracts were prepared from the magnum portion of chick oviducts as described in the Materials and methods section. Equivalent amounts (2 µg of protein) of the extract from each sample were fractionated by SDS/PAGE and transferred to nitrocellulose membranes. Immunoblots were performed with anti-OVA polyclonal antibody as described in the Materials and methods section. Only representative results are shown, and the OVA-specific band is indicated by an arrow.

analysed the levels of OVA protein in the chick oviducts of the different groups by Western blotting using a rabbit antibody specific for OVA [31]. The levels of OVA protein in chick oviducts corresponded well to the levels of OVA mRNA, therefore the OVA mRNA in chick oviduct treated with DES and TAM appeared to be translatable (lanes 3 and 4 in Figure 4). Furthermore the distribution pattern of OVA mRNA in polyribosomes examined by sucrose-gradient fractionation showed no significant difference between the chicks treated with DES and those treated with DES and TAM (results not shown).

Effect of TAM on expression of the chicken ER gene in chick oviducts

We analysed the level of the ER transcript by quantitative reverse transcription PCR [28] to determine the effect of TAM on ER gene expression. The levels of ER mRNA were constant in the chick oviducts regardless of treatment (Figure 5). Thus TAM seems to block transcription by inhibiting the transactivation function of ER, but not ER gene expression itself. The results further exclude the possibility that the inability of TAM to block the E2-induced stabilization of OVA mRNA is simply due to a decreased level of ER.

DISCUSSION

In the present study, we have shown that TAM, a synthetic E2 antagonist, completely blocks E2-induced transcription of the chicken OVA gene (Figure 1). Such loss of transcription was also observed after E2 withdrawal, in good agreement with the findings of previous studies [20,34]. The absence of E2 also caused destabilization of OVA mRNA (half-life of approx. 6 h) such that OVA mRNA could not be detected 24 h after E2 withdrawal. However, in chicks treated with E2 and TAM, the half-life of OVA mRNA was estimated to be about 24 h (Figure 2). As transcription did not occur in both groups, it appears that TAM does not inhibit E2-induced mRNA stabilization. Such E2-induced OVA mRNA stabilization was also observed when E2-induced transcription of the OVA gene was blocked by a transcription inhibitor (Figure 3). The OVA mRNA remaining

Figure 5 Effects of treatments on the level of chicken ER mRNA in chick oviducts

Chicks were treated as described in the legend to Figure 1. The transcript of the chicken ER in the chick oviducts was analysed by reverse transcription PCR as described in the Materials and methods section. (a) Representative results of DES treatment are shown. (b) The relative abundance of the ER transcript estimated from densitometric analysis is shown after normalization against β -actin mRNA and are expressed as means $\pm$ S.D. of more than five samples from different chicks. ●, DES; ○, DES + TAM; ■, WD; □, WD + TAM.

in the chicks treated with E2 and TAM for 24 h was found to be functional, as the amount of OVA protein reflected mRNA levels in all groups (Figure 4). Moreover, no alteration of ER mRNA levels by any treatment was observed in the oviducts (Figure 5). Thus, taken together, these results indicate that TAM inhibits the E2-induced transcriptional, but not post-transcriptional, regulation of chicken OVA gene expression.

Several recent studies have shown that the half-life of mRNA is controlled by cytosolic factors that bind specific sequences in its 3' untranslated region [35–37]. It has been speculated that such factors stabilize or destabilize mRNA by interacting with other factors such as RNases [37]. As steroid hormones are known to control the half-lives of mRNAs of target genes, it would be of interest to know whether nuclear hormone receptors are involved in this regulation. In the present study, we have shown that TAM does not inhibit E2-induced stabilization of OVA mRNA in chick oviducts. Taking these findings together with the fact that ER is principally located in the nucleus [38], it is unlikely that ER is involved in E2-induced mRNA stabilization. It makes sense for E2-induced post-transcriptional regulation to suggest an ER-independent E2 signalling pathway, probably mediated via other cellular and/or cell membrane receptors [39–41]. However, from the present study, we cannot exclude the possibility that TAM acts as an agonist of mRNA stabilization by permitting ER to achieve an active conformation.

TAM is a synthetic E2 antagonist that inhibits the transcription of target genes by binding to ER, and hence has been used in endocrine therapy for breast cancer patients with ER-positive tumours [16]. However, some patients with advanced disease eventually develop TAM resistance. Although a number of studies have been performed to investigate the molecular mechanism of this TAM resistance in terms of E2-induced expression of target genes, attention has been paid mainly to ER-mediated transcriptional control [42,43]. In contrast, little information is available on the mechanism of E2-induced post-transcriptional regulation. The results of the present study clearly demonstrate that, for the chicken OVA gene, TAM does not inhibit E2-induced mRNA stabilization. As post-transcriptional regulation plays a critical role in the expression of oncogenes [44,45], the possibility is raised that TAM resistance is caused by the continuous effects of E2 on gene expression at the post-transcriptional level during TAM treatment.

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