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Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter

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Using a transient co-transfection system we have identified a functional oestrogen responsive element (ERE) in the near vicinity of the TATA box of the chicken ovalbumin gene promoter. Oestrogen induced activation of transcription was observed only in chicken embryo fibroblast cells, and not in human HeLa cells, suggesting the existence of a cell-specific transcription factor(s) that is necessary for the hormonal response. By 5'-deletion mapping and site-directed mutagenesis, a sequence encompassing the more proximal of two GGTCA half-palindromic motifs was identified as a functional ovalbumin gene ERE. Both motifs correspond to oestrogen receptor binding sites *in vitro* as revealed by DNase I footprinting. Optimal response to the hormonal stimulus apparently requires stereoalignment of the ERE and the TATA box.

Key words: cell-specific oestrogen induction/oestrogen responsive element/half-palindromic motif/ovalbumin promoter/TATA box

Introduction

One of the major mechanisms by which initiation of transcription is modulated in eukaryotes involves the initial binding of nuclear proteins to specific DNA sequences usually located in the 5'-region of target genes. Steroid hormone receptors belong to such a class of DNA-binding proteins, which may act as *trans*-acting enhancer factors (for reviews and references see Yamamoto, 1985; Green and Chambon, 1987; Kumar *et al.*, 1987; Webster *et al.*, 1988; Metzger *et al.*, 1988). Steroid hormone–receptor complexes stimulate the transcription of hormonally regulated genes by binding to specific promoter DNA elements, referred to as hormone responsive elements (HRE). It has been demonstrated that such elements in glucocorticoid, progesterone and oestrogen responsive genes exhibit the properties of hormone inducible enhancers (for references see Yamamoto, 1985; Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Kumar *et al.*, 1987; Gronemeyer *et al.*, 1988). A structural and functional analysis of the promoter regions of various oestrogen regulated genes has revealed a common 13 bp-palindromic oestrogen responsive element (ERE) with the consensus sequence 5'-GGTCANNNT GACC-3' (Klein-Hitpass *et al.*, 1986; Strähle *et al.*, 1987). However, it has been shown that 'imperfect' palindromic EREs may also act synergistically to permit oestrogen induction (Martinez *et al.*, 1987). Furthermore, cooperative action of HREs with other transcription factor binding sites has been reported in the case of the tryptophan oxygenase gene (Schüle *et al.*, 1988).

The expression of the chicken ovalbumin gene is controlled *in vivo* at the transcriptional level by all four classes of steroid hormones, oestrogens, progestins, glucocorticoids and androgens (for references see Palmiter *et al.*, 1978; LeMeur *et al.*, 1981). In previous studies, we analysed the hormonal regulation of the ovalbumin gene by nuclear microinjection of recombinant plasmids, containing the ovalbumin 5'-flanking promoter sequences fused to the SV40 T-antigen coding sequences (as reporter gene), into oviduct tubular gland cells (Gaub *et al.*, 1987; Dierich *et al.*, 1987). These studies revealed that both negative and positive control elements are present in the ovalbumin promoter. Whereas the negatively acting sequences were located in the region between −425 and −132 (Gaub *et al.*, 1987), positively acting promoter sequences that were sufficient to confer cell-specific expression on the fusion gene appear to be located in close proximity to the TATA box (Dierich *et al.*, 1987). It was not possible, however, to define HREs with this system, either because of the relatively high constitutive transcription activity of the ovalbumin promoter in tubular gland cells in the absence of the negative regulatory element and/or because of the saturability of the immunofluorescence assay used in the microinjection studies. However, no sequences corresponding to any of the consensus HREs are present within 2 kb upstream of the ovalbumin gene cap site (Heilig *et al.*, 1982; L.Tora, unpublished results). Since the localization of the HREs present in the ovalbumin gene is a prerequisite for understanding the hormonal regulation of this gene at the molecular level, we have looked for sequences necessary for oestrogen induction using a transient co-transfection system. We demonstrate here that a motif corresponding to the 5' half of a consensus palindromic ERE, which is located between positions −47 and −43 of the ovalbumin promoter, is a functional ERE. However, to be active, this ERE requires cell-specific factor(s) which are expressed in chicken embryonic fibroblasts (CEFs), but not in human HeLa cells. In addition the spacing between the GGTCA motif and the TATA box appears to be important for optimal oestrogen response. Interestingly, a second half-palindromic GGTCA motif centered around position −75 is unable to mediate oestrogen induction under our present assay conditions, even though *in vitro* footprinting with DNase I indicates that both half-palindromic motifs can bind oestrogen receptor.

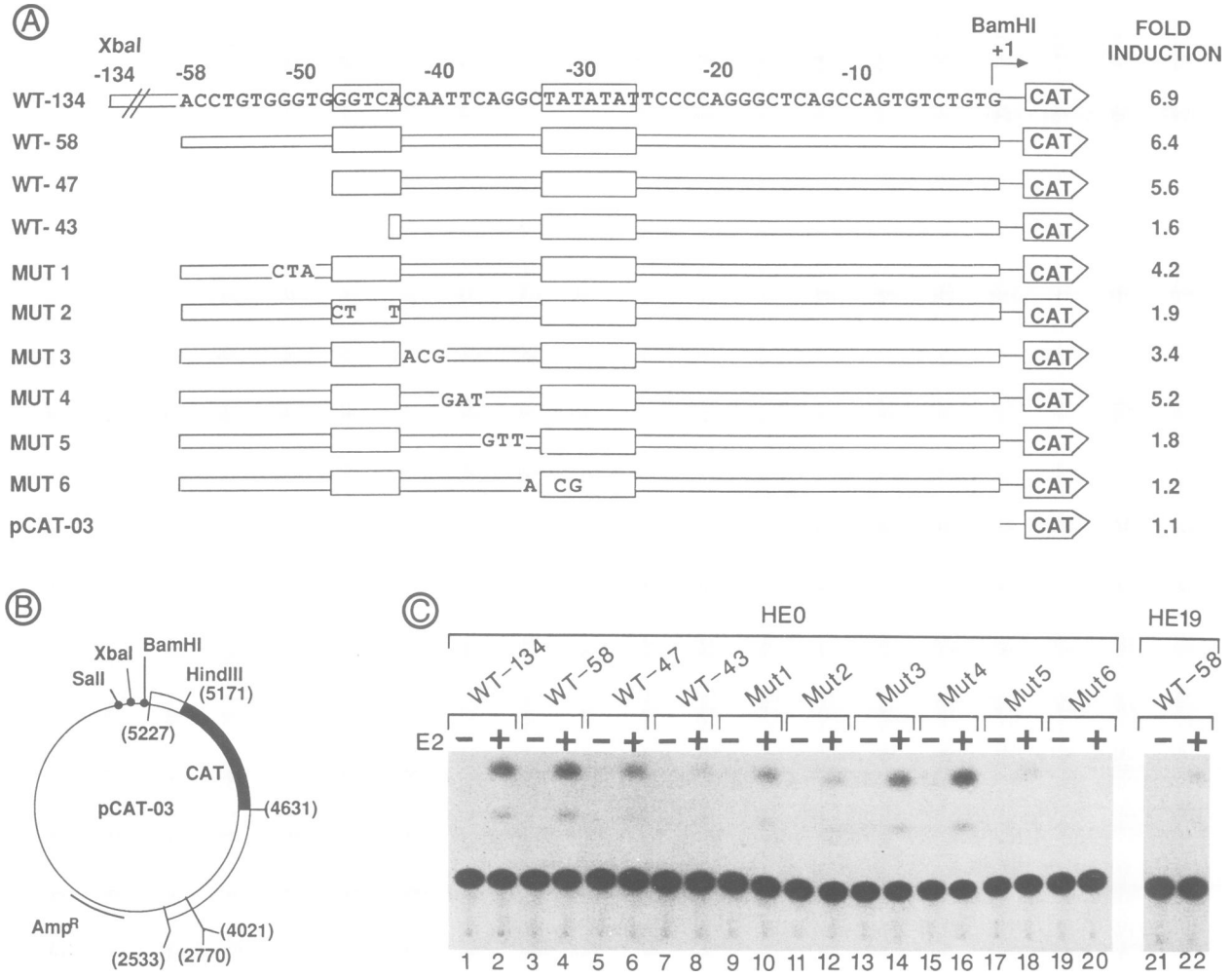

Fig. 1. Localization of the ovalbumin ERE. (**A**) Chimeric plasmids containing the ovalbumin gene 5′-flanking sequences. The GGTCA half-palindrome (see Results) and the TATA box sequences are represented as boxes. The nucleotide changes in the mutations (MUT) compared to the wild-type sequence are shown at their respective positions. Construction details are given in Materials and methods. (**B**) The promoterless CAT reporter gene containing vector pCAT-03. The ovalbumin promoter sequences were inserted between the *Xba*I and *Bam*HI sites of this vector. The black box section represents the CAT gene and the empty box illustrates the SV40 sequences with their appropriate coordinates (Tooze, 1982). (**C**) CEF cells were co-transfected with the plasmids shown in panel (A), together with the oestrogen receptor expression vector HEO (**lanes 1–20**) or HE19 (**lanes 21** and **22**). Cultures were maintained for 48 h in the absence (−) or in the presence (+) of 10 nM oestradiol (E_2) and then assayed for CAT enzyme activity with β-galactosidase calibration as described in Materials and methods. The results (shown in panel A) are expressed as fold-inductions over the control for each construction; they correspond to a minimum of three independent transfections with two independent plasmid preparations (the individual values were within ±15%).

Results

The proximal region of the ovalbumin promoter contains a cell-specific oestrogen response element

We have analysed the activity of the proximal region of the ovalbumin promoter using an assay system based on the transient transfection of primary CEFs (see Materials and methods). CEF cells do not contain any detectable endogenous oestrogen receptor, but a protein indistinguishable from the human oestrogen receptor (hER) is synthesized in CEF cells transfected with the hER expression vector HEO (Green *et al.*, 1986; Kumar *et al.*, 1987) as analysed by hormone binding and immunoblotting (data not shown). We co-transfected HEO and a fusion gene containing the ovalbumin promoter sequences between −134 and −1 in front of the promoterless bacterial chloramphenicol acetyl transferase (CAT) indicator gene (see WT-134 in Figure 1A). In the presence of 10 nM oestradiol we observed a 6.4-fold induction of CAT activity (Figure 1C, compare lanes 1 and 2), whereas no activity was detected with the promoterless vector pCAT-03 (Figure 1A and B) irrespective of the presence or absence of hormone. Similarly, no *trans*-activation of transcription from the WT-134 reporter gene was observed when HEO was omitted from the transfection. We conclude that an element is present in this −134 to −1 fragment of the ovalbumin promoter that confers oestrogen responsiveness onto CAT expression.

In order to localize the ERE by 5′-deletion mapping, we joined the previously described fragments of the ovalbumin promoter (Dierich *et al.*, 1987) to the CAT reporter gene as described in Materials and methods (see Figure 1A). The CAT reporter plasmids containing 58 bp (WT-58) or 47 bp (WT-47) upstream of the ovalbumin gene cap site were still induced between 5- and 7-fold by the oestrogen receptor in the presence of oestradiol (Figure 1A and C, lanes 3,4 and 5,6), but no significant activation above the background was

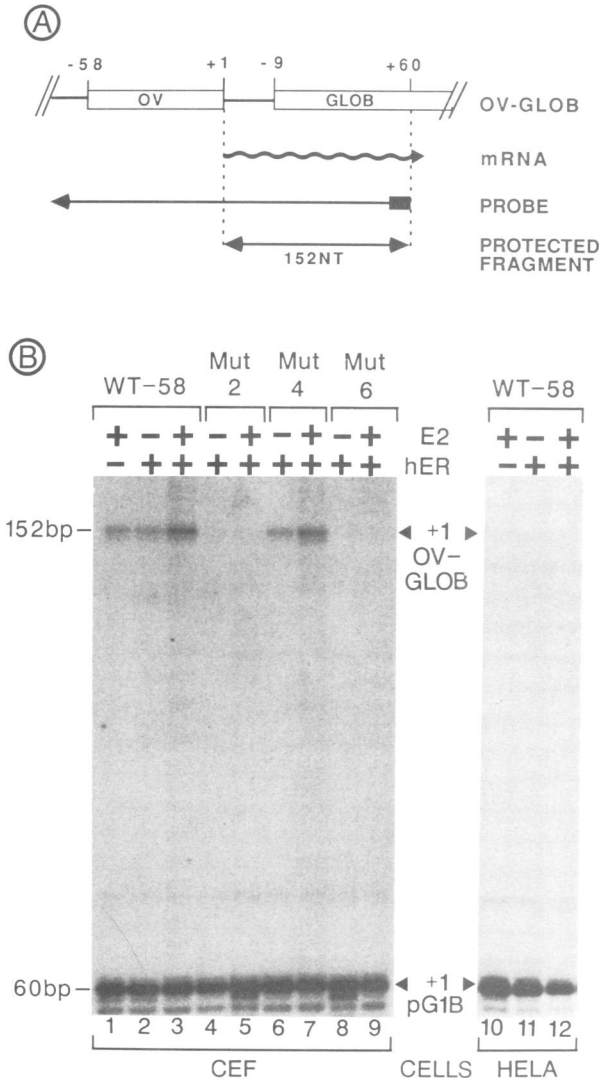

Fig. 2. Quantitative S1 nuclease analysis of oestradiol induced transcription of the OV-GLOB constructions. (**A**) Schematic illustration of the S1 nuclease mapping analysis. After hybridization of the 5′ end-labelled, single-stranded probe to RNA initiating at +1 of the OV-GLOB and treatment with S1 nuclease, the probe yields a 152 bp long protected fragment. (**B**) CEF (**lanes 1–9**) and HeLa cells (**lanes 10–12**) were co-transfected with the plasmids indicated (see also Figure 1A), with (+) or without (−) the hER expression vector along with pG1B as internal reference plasmid. Cells were maintained for 44–48 h in the absence (−) or in the presence (+) of 10 nM oestradiol (E_2) and cytoplasmic RNA was prepared. Quantitative S1 nuclease analysis was carried out using 10–15 μg cytoplasmic RNA with an excess of probe. Arrowheads represent the protected fragments, 152 bp for OV-GLOB and 60 bp for pG1B (see Materials and methods for details). Individual lanes are numbered at the bottom. Densitometric determination of the signals shown in **lanes 3** and **7** indicated a stimulation of 3.2- and 3-fold compared to **lanes 2** and **6**, respectively, after correction for the transcription from the internal control pG1B. Similar results were obtained in several independent transfections.

detected when using a CAT vector containing the first 43 bp (WT-43) of the ovalbumin promoter region (Figure 1A and C, lanes 7 and 8). These results suggest that sequences downstream of the ovalbumin TATA box are not involved in oestrogen regulation. We confirmed this conclusion by using the OV-Ad-CAT reporter gene, a recombinant which contains the ovalbumin promoter sequences from −58 to −34 located upstream of the adenovirus major late promoter sequence from −33 to +1, which itself is placed in front of the promoterless CAT gene (see Figure 3A). When co-transfected into CEF cells together with HEO, we observed a 6-fold stimulation of transcription from this reporter gene in the presence of oestradiol (Figure 3A and B, lanes 13 and 14). The sequences of the ovalbumin gene between −29 and +1 are not involved in oestrogen-dependent stimulation of transcription [note that there is a 7 bp sequence identity between the −35 and −29 sequence of the ovalbumin promoter and the −34 and −28 of the adenovirus major late promoter (Gannon *et al.*, 1979)].

The sequences between −134 and −1 of the ovalbumin gene contain two GGTCA motifs, resembling a 'half-palindrome' of the consensus ERE (see Introduction), located around positions −75 and −45 (see Figures 1A and 3A). From our 5′-deletion mapping analysis it was apparent that the motif proximal to the TATA box might correspond to a major functional ERE in our assay system. We further investigated the functional significance of this motif by mutational analysis of the WT-58 CAT vector, using the reporter recombinants Mut-1 to Mut-6 (Figure 1A). Whereas mutations in the direct vicinity of this half-palindrome only moderately affected oestrogen responsiveness (Figure 1A and C, see data for Mut-1, Mut-3 and Mut-4), a mutation of this motif to CTTCT resulted in a marked decrease of inducibility (Mut-2 in Figure 1A and C and Mut-7 in Figure 3A and B) which was marginally higher than that of the parental vector pCAT-03. As expected from our previous study (Gaub *et al.*, 1987), a mutation of the TATA box (Mut-6) was highly deleterious to the promoter activity (Figure 1A and C, lanes 19, 20). Interestingly, Mut-5 which changes sequences immediately upstream of the TATA box was also deleterious to promoter activity (Figure 1A and C, lanes 17, 18) suggesting that the GC-rich homologies, which extend three or four nucleotides upstream of several TATA boxes (Gannon *et al.*, 1979), also have an important role in transcription initiation. Based on these results, we conclude that there are two regions in the ovalbumin gene promoter which are essential for *trans*-activation of transcription by the oestrogen receptor complex, one of which is centered around the proximal GGTCA 'half-palindrome' and the other which includes the TATA box and sequences located immediately upstream.

To verify that the oestrogen induced stimulation of CAT expression was due to *trans*-activation of transcription by the ovalbumin promoter, we carried out a S1 nuclease mapping analysis of the RNA transcripts. We substituted the CAT gene in our reporter recombinants with the rabbit β-globin coding sequence, which yielded a series of OV-GLOB reporter genes, as described in Materials and methods. Following transient transfection into CEF cells, cytoplasmic RNA initiating at +1 of the OV-GLOB series was determined by quantitative S1 nuclease analysis using a [^{32}P]5′ end-labelled probe as schematically illustrated in Figure 2A (see Materials and methods). A protected DNA fragment of 152 nucleotides is expected for transcripts correctly initiated from the ovalbumin promoter. An oestrogen-insensitive reference plasmid, pG1B (see Kumar *et al.*, 1987), served as an internal control in these transfections. Figure 2B shows a 3.2- to 4-fold stimulation in the level of correctly initiated transcripts in the presence of oestradiol when the hER expression vector HEO was

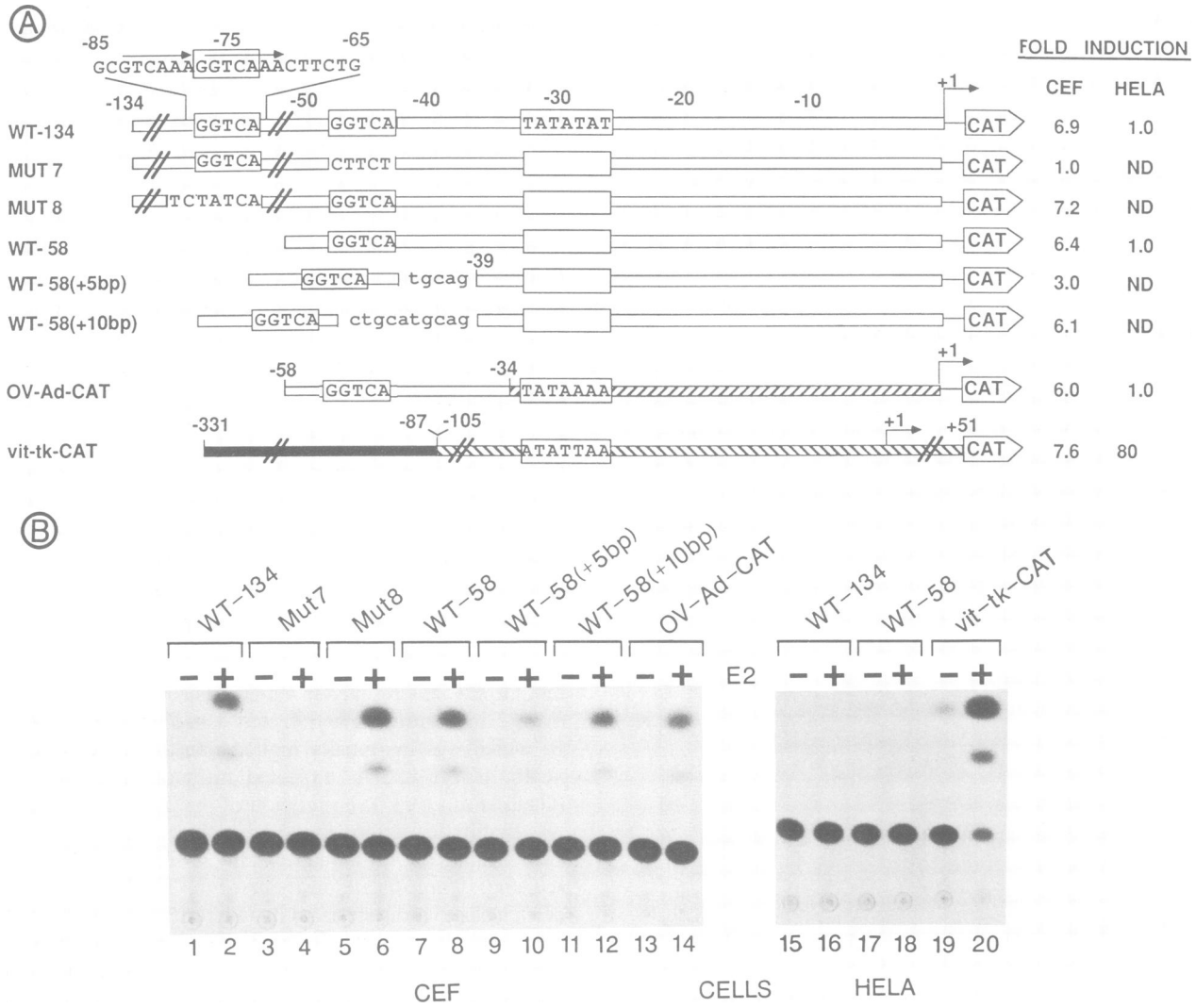

Fig. 3. (**A**) Schematic illustration of the chimeric constructions used to further characterize the ovalbumin ERE. Ovalbumin promoter sequences are shown as open lines, the adenovirus major late promoter sequence is represented as a hatched box in OV-Ad-CAT; vit-tk-CAT was described by Klein-Hitpass *et al.* (1986). The GGTCA half-palindromic motifs and the TATA box sequences are boxed. When the GGTCA motifs are mutated (Mut-7 and Mut-8) the nucleotide changes are shown at their respective positions. The COUP factor binding sites (Bagchi *et al.*, 1987) are overlined with arrows in WT-134. The lower case letters in WT-58(+5) and WT-58(+10) denote the inserted nucleotides. For details see Materials and methods. (**B**) CEF (**lanes 1–14**) and HeLa cells (**lanes 15–20**) were transfected with the plasmids shown in (**A**) together with the hER expression vector. The cells were maintained for 48 h in the absence (−) or in the presence (+) of 10 nM oestradiol (E_2) and then assayed for CAT activity with β-galactosidase calibration. The results (see panel A) are expressed as fold-inductions over the control for a minimum of three separate transfections.

co-transfected with the WT-58 OV-GLOB in CEF cells (lane 3). In the absence of oestradiol or when HEO was omitted from the transfection we detected only a basal level of transcription from WT-58 OV-GLOB (lanes 1 and 2). In keeping with the results described above for the CAT constructs, no transcripts initiating at +1 OV-GLOB were detected in the presence of co-transfected HEO and oestradiol when the GGTCA motif (Mut-2) or the ovalbumin TATA box (Mut-6) were mutated in the reporter recombinants (see Figure 2, lanes 4,5 and 8,9) whereas the mutation present in Mut-4 did not impair oestradiol induction (lanes 6 and 7). The basis for the lower constitutive transcriptional activity seen with Mut-2 OV-GLOB is unknown. We conclude that both the proximal half-palindrome and the TATA box elements are crucial in the oestrogen-receptor complex mediated activation of transcription from the ovalbumin promoter.

Only the proximal of the two half-palindromic GGTCA motifs in the ovalbumin gene promoter is a functional ERE in CEF cells, but both have the potential to interact with the DNA binding domain of the oestrogen receptor in vitro

The results obtained with the reporter genes WT-134 and WT-58 indicated that the sequences between −58 and −134 did not contribute to oestrogen responsiveness in our system. This region of the ovalbumin promoter exhibits sequence homology to the corresponding regions of various other genes (Benoist *et al.*, 1980) and has been postulated to be essential for efficient initiation of transcription from the ovalbumin promoter (Knoll *et al.*, 1983; Pastorcic *et al.*, 1986). Furthermore the sequences surrounding the distal GGTCA motif appear to be even more important as they have been shown to be the binding site of the so-called COUP (chicken ovalbumin upstream) factor (see Figure 3A,

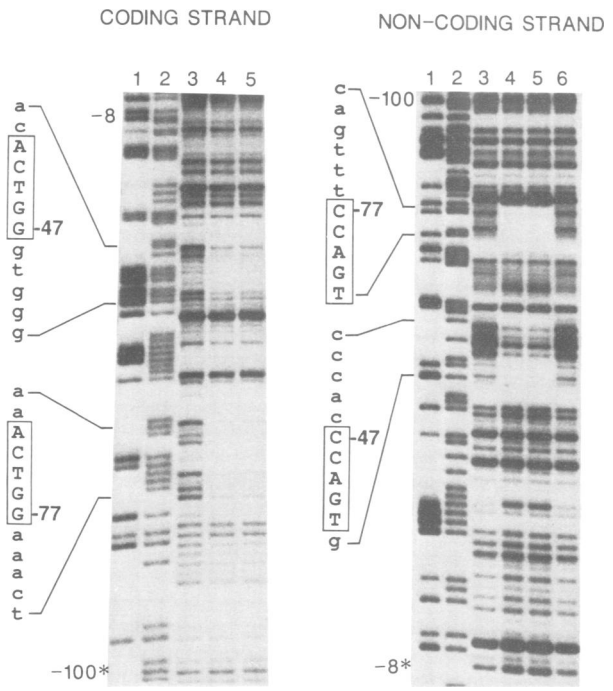

Fig. 4. DNase I protection assay on the ovalbumin promoter using a fusion protein containing the DNA binding domain of the human oestrogen receptor. The experiment was carried out with [^{32}P]5′ end-labelled ovalbumin promoter fragments as described in Materials and methods. G- (**lanes 1**) and G+A-specific (**lanes 2**) sequence ladders. **Lanes 3**: pattern of protection in the presence of 1 μg of β-galactosidase control protein preparation. **Lanes 4** and **5**: pattern of protection with 1 μg of fusion protein extract containing the oestrogen receptor DNA binding domain. **Lane 6**: DNase I digestion of naked DNA. The proximal and distal protection sites are shown on both strands, with the half-palindromic GGTCA motifs boxed.

overlined with arrows in WT-134) (Bagchi *et al.*, 1987). In the Mut-8 CAT reporter gene the sequence AAGGTCA was changed to TCTATCA (Figure 3A), thus affecting both the GGTCA half-palindrome and the COUP factor binding sites. In the presence of co-transfected HEO and oestradiol, Mut-8 CAT showed a 7.2-fold induction of CAT activity as compared with a 6.9-fold induction generated by the wild-type WT-134 (Figure 3A and B). In contrast no hormonal induction was seen with Mut-7 (Figure 3A and B), which carries the Mut-2 (Figure 1A) mutation in the proximal half-palindromic motif.

To investigate whether the activation characteristics of the GGTCA motifs could be related to their differing distances from the ovalbumin gene TATA box, two WT-58-based CAT reporter genes were constructed in which the proximal motif was spaced by five or ten additional base pairs from the TATA box. Interestingly, the addition of 10 bp (i.e. approximately one DNA helical turn) in WT-58(+10) did not affect the inducibility of the recombinant by the oestrogen receptor complex, whereas the addition of 5 bp in WT-58 (+5) reduced the induction by ~50% (Figure 3A and B, compare lanes 7,8 and 11,12 with lanes 9,10). These data indicate that there is no absolute requirement for a precise position of the GGTCA motif with respect to the TATA box, but suggest that 'cooperation' of the factors is optimal when they are stereoaligned along the DNA helix.

To examine the possible role of the hER A/B region in activation of transcription from the ovalbumin promoter in CEF cells, we co-transfected WT-58 and HE19 (Kumar *et al.*, 1986), which expresses a truncated hER lacking the entire A/B region. Irrespective of the presence or absence of hormone, no significant *trans*-activation of transcription was observed (see Figure 1C, lanes 21 and 22), which indicates that the A/B domain of the receptor is required directly or indirectly for *trans*-activation of the ovalbumin promoter.

To investigate further the molecular basis for the oestrogen inducibility, we analysed the potential of the ovalbumin promoter region to bind the oestrogen receptor *in vitro*. To this end we used the bacterially expressed DNA binding domain of the hER, produced as a fusion protein with β-galactosidase (see Materials and methods). The results obtained by DNase I footprinting with the purified fusion protein are shown in Figure 4 (see lanes 4 and 5). A clear protection of two regions was observed encompassing both proximal and distal GGTCA motifs on both the coding and the non-coding strands. No protection was seen in the presence of purified β-galactosidase (Figure 4, lanes 3) or when no protein was added (lane 6). Therefore, *in vitro* both half-palindromic motifs can bind the DNA binding domain of the oestrogen receptor, although only the more proximal one is functional as an ERE *in vivo*.

The ovalbumin ERE is not functional in HeLa cells

Oestrogen receptor complexes binding to palindromic EREs are able to induce heterologous promoters in various vertebrate cell types (for references see Druege *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Kumar *et al.*, 1987; Green *et al.*, 1988) and in yeast (Metzger *et al.*, 1988) indicating that in these systems they do not require additional cell-specific factors to activate the corresponding reporter genes. Thus, we attempted to induce the ovalbumin ERE containing recombinants in human HeLa cells, by transfecting either the rabbit β-globin (Figure 2B, lanes 10−12) or the CAT (Figure 3A and B, lanes 15−18) reporter genes, together with the hER expression vector HEO in the presence or absence of oestradiol. None of the ovalbumin promoter-containing reporter recombinants could be stimulated (Figures 2B and 3A). Surprisingly, not even the OV-Ad-CAT recombinant could be activated in HeLa cells, although it has been shown that the same TATA box containing major late promoter fragment can be activated by a palindromic ERE in these cells (unpublished results from our laboratory).

Note that the vit-tk-CAT recombinant (Klein-Hitpass *et al.*, 1986), which contains the palindromic ERE of the *Xenopus laevis* vitellogenin A2 gene located upstream of the HSV thymidine kinase promoter (used as a positive control in our experiments), could be activated by HEO and oestradiol ~10 times more efficiently in HeLa cells than in CEF cells (Figure 3A).

Discussion

The proximal half-palindromic motif of the ovalbumin promoter is a functional ERE

By transient co-transfection into CEF cells, we have identified a functional ERE located within the 58 bp upstream of the ovalbumin gene cap site. Using a similar approach we have recently analysed the distinct progesterone and glucocorticoid HREs present in the proximal promoter region

of the same gene (Tora *et al.*, 1988; and our unpublished data). To further characterize the ERE, we made a series of mutations encompassing a sequence motif identical to a 'half-palindromic' consensus ERE (Klein-Hitpass *et al.*, 1986). The results obtained with these mutations revealed that this GGTCA pentanucleotide located between position −47 and −43 has an essential role in the oestrogen-induced stimulation. Using DNase I protection assays we demonstrated that this sequence has the potential to bind the DNA binding domain of the hER (Figure 4). These results demonstrate that even a half-palindromic element can be a functional ERE.

Interestingly a second, more distal GGTCA motif is present 30 bp further upstream. Although we were able to demonstrate that this region also binds to the bacterially expressed DNA binding domain of the oestrogen receptor *in vitro*, it is not functional *in vivo*. Neither a deletion of this sequence nor a mutation of the motif affected the oestrogen inducibility of the corresponding reporter recombinants in transient co-transfection experiments. We note that this region also contains the postulated recognition sequence of the so-called COUP factor which has been proposed to be important for the transcription of the ovalbumin gene (Knoll *et al.*, 1983; Pastorcic *et al.*, 1986; Bagchi *et al.*, 1987; Tsai *et al.*, 1987) both *in vitro* and *in vivo*. However, we did not detect any positive effect of this region on the transcriptional activation of our reporter genes in CEF cells. As we have noticed that nuclear extracts of various chicken tissues contain protein(s) provoking a strong protection at this position in DNase I footprinting assays (L.Tora, unpublished results), we suggest that these protein(s) may be present in CEF cells and compete with the oestrogen receptor for the binding to the distal GGTCA element. Such a competition has been proposed to be the basis for the glucocorticoid insensitivity of the Moloney murine leukaemia virus enhancer compared with the closely related glucocorticoid inducible Mo-MSV enhancer (Speck and Baltimore, 1987).

In transiently transfected CEF cells we detected at most a 6- to 8-fold *trans*-activation of transcription from the ovalbumin gene promoter by oestradiol. However, in the chicken, oestrogen stimulation can increase the rate of ovalbumin gene transcription by about three orders of magnitude (Palmiter *et al.*, 1978; LeMeur *et al.*, 1981). Thus the stimulation we observed in CEF cells represents only one, probably elementary, aspect of the oestrogen-induced transcriptional stimulation of the ovalbumin gene. This is illustrated by the fact that upon oestrogen induction there are changes in the chromatin structure of the ovalbumin gene 5′ flanking sequences up to 7 kbp upstream of the cap site (Kaye *et al.*, 1986) suggesting that the oestrogen regulation of this gene is not only limited to the short promoter region studied here. Interestingly, in CEF cells, we detected only a minor oestrogen stimulation (~2-fold) with an OV-CAT recombinant bearing a 425 bp 5′ flanking sequence and with recombinants bearing 5′ flanking sequences longer than 425 bp we could not detect any stimulation (data not shown). It is possible that the negative regulatory element localized in our previous study to between −425 and −295 (Gaub *et al.*, 1987) is severely blocking transcription in CEF cells. In oviduct tubular gland cells this repression could be relieved in a cell-specific fashion in response to steroid hormones (Gaub *et al.*, 1987).

Activation of transcription mediated by the ovalbumin half-palindromic ERE requires cell-specific factor(s) in addition to the oestrogen receptors

In contrast to the results obtained with CEF cells, none of the ovalbumin ERE containing recombinants could be stimulated by oestradiol in HeLa cells (see Figures 2 and 3). This observation suggests the existence of a specific 'factor' in CEF cells necessary for the hormonal induction and which is not present in HeLa cells. One possibility would be that there are significant differences between the TATA box factor(s) of these transcriptional systems. Our preliminary results obtained with WT-58(+5) and WT-58 (+10) indicate a requirement for a stereoalignment of the TATA box and ERE for optimal stimulation. This may reflect a direct or an indirect interaction (mediated by a cell-specific factor) between the oestrogen receptor and the TATA box factor or some other element of the basic transcription machinery bound to this factor. Note that we did not observe any significant changes in the oestrogen inducibility using OV-Ad-CAT construction where the ovalbumin TATA box sequences (TATATAT) were replaced with the corresponding Ad2 MLP sequences (TATAAAA) (see Figure 3, WT-58 and OV-Ad-CAT), suggesting that the TATA factor(s) interact similarly with both sequences. However, the possible existence of multiple TATA-specific factors was proposed from studies of the E1A dependent regulation of the hsp70 promoter (Simon *et al.*, 1988). Thus, it is possible that different cells express different sets of TATA-specific factors which might play an important role in cell-specific gene regulation. This present cell specificity and cooperativity for hER function has not been observed previously with promoters associated with palindromic EREs (Kumar *et al.*, 1987). A half-palindromic ERE binds the hER only very weakly when compared with the binding to a palindromic ERE (Kumar and Chambon, 1988; Mader *et al.*, 1988), which raises the possibility that the binding of the hER to a half-palindromic motif may be stabilized *in vivo* by interactions with other cell-specific factor(s). It is possible that such interactions are mediated by the receptor's N-terminal A/B region, since no activation of the ovalbumin ERE could be detected using the hER mutant HE19, which lacks this region (see Figure 1C, lanes 21 and 22). In this respect note that both HEO and HE19 will fully activate in HeLa cells the strongly oestradiol-induced promoter 'vit-tk', which contains a perfect palindrome (Kumar *et al.*, 1987). Conversely, the pS2 gene promoter, which contains a weaker imperfect ERE palindrome (Berry *et al.*, 1988), is induced by HE19 to only 17% of the level observed with HEO (Kumar *et al.*, 1987). We note in addition that the glucocorticoid and progesterone inductions of the ovalbumin gene promoter exhibit cell-specificity (Tora *et al.*, 1988), which suggests that their receptors may also cooperate with cell-specific factor(s).

Examples of palindromic HREs tightly clustered with additional transcriptional factor binding sites have been described for the 5′ flanking sequences of other hormone-responsive genes, for example the chicken lysozyme (Steiner *et al.*, 1987), the human metallothionein IIA (Lee *et al.*, 1987) and the rat tryptophan oxygenase gene (Danesch *et al.*, 1987). Protein−protein interactions have been postulated for the glucocorticoid receptor and the CACCC box binding transcription factor in the tryptophan oxygenase gene (Schüle *et al.*, 1988). Such protein−protein interaction, generating

synergistic effects, may be one of the molecular mechanisms involved in hormone-dependent activation of transcription. In this respect, the ovalbumin gene promoter might be a particularly good system to analyse these mechanisms since the transcriptional activity of the hER bound to a half-palindromic motif is fully dependent on the presence of additional cell-specific factor(s).

Materials and methods

Construction of reporter plasmids

The plasmid pCAT-03 was constructed by replacement of the *SalI*−*Hin*dIII fragment of the chicken collagen 2 promoter region from pCOL-CAT (Herbomel *et al.*, 1984) with a *SalI*−*Hin*dIII fragment. This fragment contained a polylinker with *Sal*I, *Xba*I, *Bam*HI sites and SV40 sequences from position 5227 to 5171 (Tooze, 1982) as a result of the cloning strategy (see Figure 1B). The WT-134 OV-CAT plasmid was made by excising the ovalbumin promoter sequences with *Xba*I and *Bam*HI from the previously described plasmid pTOT-134 (Dierich *et al.*, 1987) and inserting them into the corresponding sites pCAT-03. The WT-58, WT-47, all the mutated ovalbumin fragments (shown in Figure 1A) and the OV-Ad fragment (shown in Figure 3A) were constructed using synthetic oligonucleotides with *Xba*I and *Bam*HI ends. The corresponding single-stranded oligonucleotides were annealed (by incubation for 1 min at 90°C, followed by a further 30 min at room temperature in 10 mM Tris−HCl pH 8 containing 5 mM $MgCl_2$) and cloned into the *Xba*I−*Bam*HI sites of pCAT-03. The WT-58 OV-GLOB plasmid has been described previously (Tora *et al.*, 1988). The mutated ovalbumin fragments were cloned in this plasmid by exchanging the wild-type sequences with the appropriate mutations (Figure 2B). Mutations 7 and 8 and the insertions (shown in Figure 3A) were prepared by site-directed mutagenesis using double-stranded plasmid DNA (Inouye and Inouye, 1987). All the constructions were verified by sequencing. In all the ovalbumin promoter-containing plasmids the G at position +1 replaces A in the wild-type sequence. The vit-tk-CAT plasmid (Klein-Hitpass *et al.*, 1986) was kindly provided by G.Ryffel.

Cell transfections and CAT assay

CEF primary tissue cultures were prepared from 9−11-day-old embryos according to Solomon (1976). We used CEF cells because these cells could be more efficiently transfected than other primary chicken cells when using the classical calcium phosphate precipitation technique (see below). CEF and HeLa cells were maintained in Dulbecco's modified Eagle's medium without phenol red supplemented with 5% fetal calf serum which had been treated with dextran-coated charcoal to remove traces of steroid hormones (Dierich *et al.*, 1987). Cells were transfected at 40−60% confluence in 6 cm Petri dishes using the calcium phosphate precipitation technique (Banerji *et al.*, 1981). For CAT assays, 200 ng of vit-tk-CAT or 5 μg of the other reporter plasmids and 500 ng of the hER expression vector HEO or HE19 (Green *et al.*, 1986; Kumar *et al.*, 1986, 1987) were used together with 1 μg of the pCH110 (Pharmacia) β-galactosidase expression vector, used as an internal control to normalize for variations in transfection efficiency. The total amount of DNA transfected was made up to 10 μg with carrier DNA (Bluescribe M13+). When required, 10 nM oestradiol was added to the medium. CAT assays were carried out as described by Webster *et al.* (1988).

RNA preparation and quantitative S1 nuclease mapping analysis

CEF and HeLa cells were transfected as above, using 10 cm Petri dishes, 10 μg of reporter plasmids, 1 μg of HEO and 400 ng of the reference plasmid pG1B (Kumar *et al.*, 1987). Carrier DNA (Bluescribe M13+) was added to 20 μg. Cells were harvested 44−48 h after transfection and cytoplasmic RNA was isolated as described (Groudine *et al.*, 1981). Single-stranded DNA probes were prepared by primer extension of single-stranded M13 DNA templates containing the 1301-bp long *Eco*RI−*Eco*RI fragment from OV-GLOB (see constructions). The primer used is complementary to bases +39 to +60 of the rabbit β-globin gene (Zenke *et al.*, 1986) and was 5′ end-labelled with [γ-^{32}P]ATP prior to extension. The labelled DNA was cut with *Eco*RI and the single-stranded probe was isolated from a 5% strand separating gel according to Maniatis *et al.* (1982). Quantitative S1 nuclease analysis was carried out as described by Kumar *et al.* (1987).

DNase I footprinting experiments

An *Escherichia coli* expressed β-galactosidase fusion protein containing the hER DNA binding domain from amino acids 175−282 was prepared as described by Mader *et al.* (1988). [^{32}P]5′ end-labelled double-stranded ovalbumin gene fragments were prepared according to Maniatis *et al.* (1982) and labelled either at position −134 (coding strand) or at position +7 (non-coding strand). Labelled DNA (10 000 c.p.m.) was incubated with or without 1 μg of the appropriate protein preparations at 0°C for 20 min in 20 mM Hepes pH 7.9, 50 mM KCl, 3.5 mM $MgCl_2$, 2 mM DTT and 15% glycerol (without any competitor DNA). DNase I digestion was carried out at 20°C for 90 s with 4 ng of DNase I. The reaction was stopped by adding 200 μl of 0.3% SDS, 140 mM NaCl, 50 mM Tris−HCl, pH 8.0, 50 μg/ml tRNA. After phenol/chloroform extraction and ethanol precipitation the samples were analysed on an 8% polyacrylamide/8.3 M urea sequencing gel. G and G+A reactions were carried out as described by Maxam and Gilbert (1977).

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