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The chicken ovalbumin promoter is under negative control which is relieved by steroid hormones

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Steroid hormone regulation of activity of the chicken ovalbumin promoter was studied by microinjection of chimeric genes into the nuclei of primary cultured oviduct tubular gland cells. The chimeric genes contained increasing lengths of ovalbumin gene 5′-flanking sequences fused to the sequence coding for the SV40 T-antigen. Promoter activity was estimated by monitoring synthesis of T-antigen. The activity of the ovalbumin promoter is cell-specifically repressed in these oviduct cells and the repression is relieved upon addition of steroid hormones. The −132 to −425 region of the ovalbumin promoter which is responsible for this negative regulation behaves as an independent functional unit containing the regulatory elements necessary for both repression (in the presence of steroid hormone antagonists) and induced derepression (in the presence of steroid hormones) of linked heterologous promoters.

Key words: transcription/regulation/microinjection/oviduct tubular gland cells/primary culture

Introduction

Understanding how patterns of gene transcription are controlled both during the development of eukaryotic organisms and in terminally differentiated cells is one of the present goals of molecular biology. This control may be achieved either positively or negatively through the interaction of *trans*-acting proteins with *cis*-acting DNA promoter elements (Ptashne, 1986 and refs therein). To date, very few regulatory proteins have been characterized in higher eukaryotes. In this respect, steroid hormone receptors are of particular interest, since their ability to control initiation of transcription from specific genes in target cells is dependent on the binding of their specific ligand. In the case of genes induced by glucocorticoids, activation of transcription results from the specific binding of the hormone-receptor complex to DNA responsive elements (GREs) which exhibit the properties of inducible promoter enhancers (for review, see Beato *et al.*, 1985 and Yamamoto, 1986). Similarly, activation of transcription of the *Xenopus* (Druege *et al.*, 1986; Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986) and chicken (Jost *et al.*, 1985 and refs therein) vitellogenin genes, and of the human pS2 gene (Brown *et al.*, 1984; M.Berry, A.N.Nunez and P.Chambon, unpublished results) by oestrogens appears to involve the interaction of the cognate receptor complex with inducible, enhancer-like, responsive elements (EREs). In both cases, as well as for the activation of the chicken lysozyme gene promoter by dexamethasone and progesterone (Renkawitz *et al.*, 1984), the regulation seems to be essentially positive, in that the interaction of the hormone-receptor complex with its responsive element leads to the activation of promoter elements whose intrinsic activity is very low.

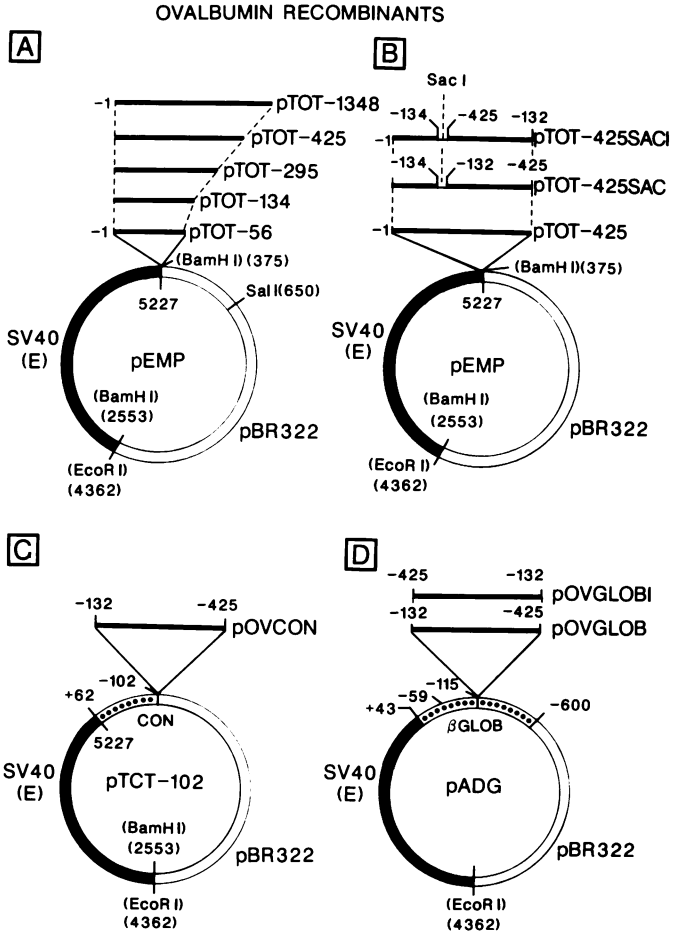

Fig. 1. Structure of chimeric ovalbumin promoter recombinants. Constructions are described in Materials and methods. Restriction sites in parentheses correspond to sites which have been lost during the cloning. Coordinates of ovalbumin and conalbumin fragments are given with respect to the RNA start site (+1). **Panel A**: a series of pTOT recombinants with insertions of different lengths of the ovalbumin promoter region (thin black line) upstream to the SV40 early coding region [SV40(E)] from positions 5227–2553 (BBB system) (thick black line). The open double line corresponds to pBR322. **Panel B**: pTOT-425SAC and pTOT-425SACI recombinants in which the ovalbumin promoter region from −132 to −425 is inserted in either orientation upstream to the −1 to −134 sequence. **Panels C** and **D**: the ovalbumin promoter sequence from −132 to −425 is inserted upstream of heterologous promoters; (**C**), pOVCON, the ovalbumin fragment is inserted in pTCT-102 immediately upstream to the conalbumin promoter region from −102 to +62 to construct the pOVCON plasmid; (**D**) the ovalbumin fragment is inserted in either orientation at positions −59 [pOVGLOB(59) and pOVGLOBI(59)] or −115 [pOVGLOB(115) and pOVGLOBI(115)] of the chicken β-globin promoter in the pADG plasmid described in the accompanying paper (Dierich *et al.*, 1987). pOVGLOB(115) and pOVGLOBI(115) only are represented here.

Table I. Effect of anti-oestrogens (Tamoxifen) and anti-glucocorticoids (RU486) on the activity of the ovalbumin promoter in primary cultures of chicken oviduct cells maintained in medium with foetal calf serum (MFCS)

Medium	Immunofluorescent cells (% of pSV1)				
	Recombinants				
	Control (pSV1)	pTOT-134	pTOT-295	pTOT-425	pTOT-1348
MFCS	100% (A)	66%[53–91](4)	53%[36–64](7)	53%[38–77](9)	53%[41–66](6)
MFCS + Tamoxifen (10^{-6} M)	100% (B)	49%[46–54](3)	47%[33–64](4)	39%[23–54](4)	n.d.
MFCS + RU486 (2×10^{-6} M)	100% (C)	47%[43–52](4)	41%[36–45](3)	26%[15–34](4)	n.d.
+ RU486 Oestradiol (10^{-8} M)	100%	n.d.	n.d.	56%[51–60](2)	n.d.
MFCS + Tamoxifen RU486	100% (D)	60%[44–84](6)	44%[36–49](4)	8%[0–16](8)	8%[5–15](6)
+ Tamoxifen, RU486 Progesterone (10^{-9} M)	100% (D)	n.d.	n.d.	50%[30–75](7)	44%[31–54](6)

The DNA of the chimeric recombinants of the pTOT series (Figure 1A) was microinjected 3 days after plating into cell nuclei at ~100 copies/nucleus. The antagonists were added immediately after the microinjection, cells were fixed 24 h after microinjection and stained by indirect immunofluorescence using T-antigen monoclonal antibodies. Fluorescent nuclei were counted and the results were expressed in percent relative to those obtained by microinjection of the SV40 control plasmid pSV1 (as described in Dierich *et al.*, 1987). The mean values are underlined, the extreme values are within square brackets and the number of independent experiments are within parentheses. 100% pSV1 controls corresponded to (A) 53%[43–59](8), i.e. on the average pSV1 microinjected cells were T-antigen positive with extreme values of 43–59% in eight experiments; (B) 61%[57–67](3); (C) 58%[54–65](4); (D) 55%[46–71](6). N.d.: not determined.

We have previously cloned and determined the structure of the ovalbumin gene whose transcription can be specifically modulated in oviduct tubular gland cells of the chicken by administration or withdrawal of oestrogens, progestins, glucocorticoids and androgens, each acting through a distinct receptor (LeMeur *et al.*, 1981 and refs therein). We have reported also that the ovalbumin gene is neither accurately nor efficiently expressed when transferred *in vitro* into a variety of non-oviduct cells (Breathnach *et al.*, 1980), whereas various fragments of the ovalbumin gene promoter are specifically active in primary cultured chicken hepatocytes and oviduct tubular gland cells (Dierich *et al.*, 1987). Furthermore, in the latter case the activity of a promoter fragment encompassing the first 425 bp located upstream to the initiation site appeared to be dependent on the presence of steroid hormones in the culture medium. In contrast to the steroid hormone-responsive genes which have been studied up to now (see above), we report here that the ovalbumin gene promoter is subjected to a negative regulation in oviduct tubular gland cells, and that the effect of steroid hormones is to relieve this cell-specific repression. We show also that the negative control element which mediates this repression can act on heterologous promoters. Preliminary accounts of this study have been reported previously (Chambon *et al.*, 1984; Gaub *et al.*, 1985).

Results

Effect of steroid hormone antagonists on the activity of the ovalbumin promoter microinjected in tubular gland cells maintained in a medium containing foetal calf serum

There is no permanent chicken oviduct cell line containing oestrogen and progestin receptors in which the ovalbumin promoter could be transferred to study its function and the mechanisms of its hormonal activation. In addition, the ovalbumin promoter is inactive when introduced in heterologous permanent cell lines which contain these receptors, like the human breast cancer cell line MCF-7 (Dierich *et al.*, 1987). Thus, we have used primary cultured cells derived from oviducts of immature chicken which have been previously treated with oestrogens. After 3 days of culture, there is still an average of approximately 5000 oestrogen receptor molecules per cell (data not shown). As mentioned in the accompanying paper (Dierich *et al.*, 1987), we have not succeeded up to now in expressing efficiently the ovalbumin promoter following DEAE-dextran or calcium phosphate DNA transfection into primary cultured tubular gland cells, unless an heterologous enhancer element, such as that of the Simian virus 40 (SV40), was also contained in the transfected recombinant. However, no hormonal response could be observed in the presence of this enhancer (our unpublished results). We have, therefore, used the nuclear microinjection method which has allowed us to study the cell-specific activity of the conalbumin and ovalbumin promoters (Dierich *et al.*, 1987). Since the RNA transcribed from the microinjected ovalbumin promoter-containing recombinant could not be routinely analysed, we have used the ovalbumin promoter recombinant series pTOT [Figure 1 and accompanying paper (Dierich *et al.*, 1987)], in which increasing lengths of 5′-flanking sequences of the ovalbumin gene have been inserted upstream to the SV40 T-antigen coding sequence, so that T-antigen expression was under the control of the ovalbumin promoter. These chimeric recombinants were microinjected into the nuclei of oviduct tubular gland cells, steroid hormones or their antagonists were added to the culture medium, and 24 h later the activity of the ovalbumin promoter fragments was estimated by scoring the number of fluorescent nuclei following an indirect immunofluorescence reaction against T-antigen (Moreau *et al.*, 1981; Dierich *et al.*, 1987). In each series of experiments, the results were expressed as a percentage relative to the number of immunofluorescent cells obtained by microinjecting the wild-type SV40 early region recombinant pSV1 (Benoist and Chambon, 1980), whose promoter functions in all cell types and is not sensitive to steroid hormones (Dierich *et*

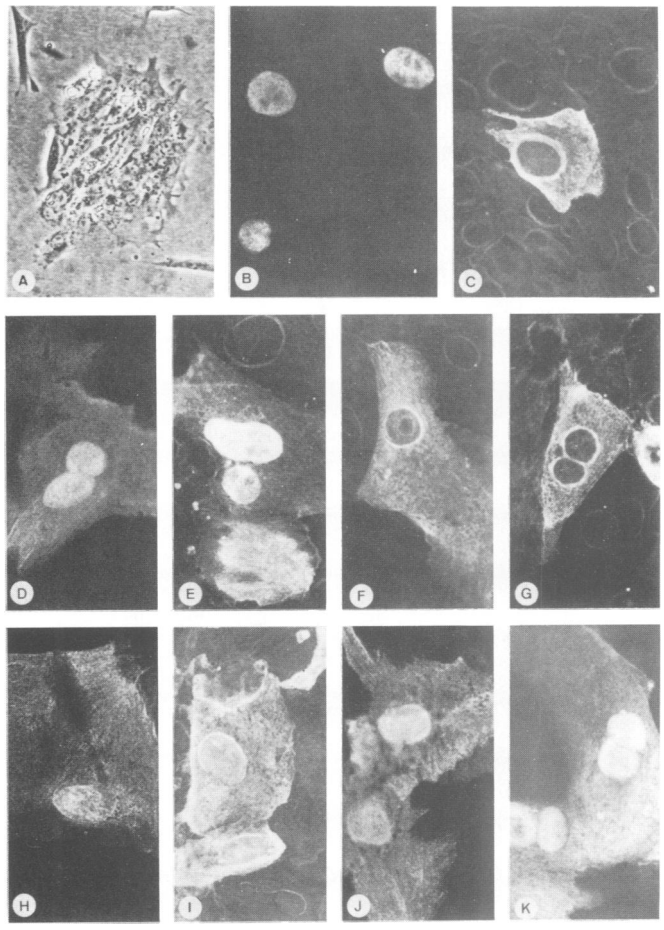

Fig. 2. Effects of steroid hormone antagonists (Tamoxifen and RU486) on the activity of the ovalbumin promoter recombinants pTOT-134 and pTOT-425. pTOT-134 and pTOT-425 were coinjected with a SV40 control recombinant D10 [producing only cytoplasmic SV40 T-antigen, for details see text and the accompanying paper of Dierich *et al.* (1987)] into chicken oviduct cells maintained in a medium containing foetal calf serum (MFCS). (**A**) Phase contrast micrograph (× 125) of primary cultured chicken oviduct cells. The other micrographs (× 500) show primary cultured oviduct tubular gland cells in which SV40 T-antigen synthesis is detected by indirect immunofluorescence. In (**B**) and (**C**), the cells were injected with pTOT-425 (**B**) (a similar pattern is obtained with pTOT-134, not shown) or D1O(**C**). D10 and pTOT-425 were co-microinjected in cells maintained in MFCS in the absence (**D**−**E**) or in the presence of both antagonists (**F**−**G**). D10 was co-microinjected with pTOT-134 in cells maintained in MFCS in the absence (**H**−**I**) or in the presence of both antagonists (**J**−**K**). Microinjection was performed as described in Dierich *et al.* (1987) and in Table I, except that pTOT-425 and pTOT-134 were microinjected at ~200 DNA copies/nucleus, whereas D10 was microinjected at 20 DNA copies/nucleus. Antagonists were added immediately after the microinjection (at the same concentration as in Table I).

al., 1987; usually 50−60% of the nuclei of cells microinjected with pSV1 were fluorescent, see tables).

All five recombinants pTOT-56, pTOT-134, pTOT-295, pTOT-425 and pTOT-1348 (Figure 1A) were expressed at approximately the same level after microinjection into nuclei of tubular gland cells maintained in a medium containing 10% foetal calf serum (MFCS) (Table I; the results for pTOT-56, which in all cases were identical to those obtained with pTOT-134, are not shown in this table or in the other tables). In contrast, and in agreement with the results shown in the accompanying study (Dierich *et al.*, 1987), none of the pTOT recombinants are expressed when microinjected into the fibroblasts which are also present in primary cultures of chicken oviduct cells (not shown). That there were fewer tubular gland cells expressing the pTOT recombinants than the SV40 control pSV1, reflects most probably a heterogeneity in these cells, since doubling the amount of microinjected pTOT DNAs did not result in significant increases in the number of fluorescent cells (not shown).

Since glucocorticoids, oestrogens and progestins are present in the medium containing 10% FCS at approximately 10^{-10} to 10^{-11} M, we investigated next the effect of the anti-oestrogen Tamoxifen and of RU486, which is both an anti-glucocorticoid and an anti-progestin in mammals (Philibert *et al.*, 1981) but exhibits only anti-glucocorticoid activity in chickens (Groyer *et al.*, 1985). The simultaneous addition of Tamoxifen and RU486 did not significantly affect the expression of pTOT-56 (not shown), pTOT-134 and pTOT-295, whereas the expression of pTOT-425 and pTOT-1348 was drastically inhibited. The residual expression of these two recombinants may be related to the presence of variable amounts of progestins in the culture medium since addition of progesterone to the medium containing both inhibitors restored the activity of the ovalbumin promoter to its original level (Table I). It may also reflect some 'leaky' transcription, not initiated under the control of the ovalbumin promoter, since we have observed that the reduced activity of the TATA box-mutated promoter present in pTOT-425 M (see Dierich *et al.*, 1987) is much less sensitive to the addition of antagonists than that of the wild-type pTOT-425. It is interesting to note that the addition of progesterone for 24 h to the tubular gland cells maintained in the presence of Tamoxifen and RU486 exerted a similar stimulation on the expression of the endogenous ovalbumin gene, since a 3- to 10-fold increase in ovalbumin mRNA can be observed (F.Bellard, personal communication), in agreement with the results obtained by Jung-Testas *et al.*, (1986). When Tamoxifen or RU486 are added separately to the culture medium, the expression of pTOT-425 is decreased moderately, whereas no significant change is observed for pTOT-134 or pTOT-295, suggesting that both oestrogen and glucocorticoids contribute to the activity of the ovalbumin promoter of pTOT-425. The possible dependence on oestrogens was further supported by the observation that the original activity is restored when oestradiol is added together with RU486 (Table I).

The number of fluorescent nuclei did not appear to decrease significantly in tubular gland cells when maintained in culture in the presence of both Tamoxifen and RU486 and microinjected with the SV40 control recombinant pSV1. However, it was important to demonstrate that the decreased expression of pTOT-425 in the presence of these inhibitors was not due to the disappearance of the responsive cells. This possibility was directly ruled out by using a SV40 recombinant similar to pSV1 but encoding the T-antigen mutant D10 (Kalderon *et al.*, 1984), which is not translocated to the nucleus. As expected, T-antigen was localized in the nucleus when tubular gland cells, maintained in MFCS, were microinjected with pTOT-425 (Figure 2, panel B), pTOT-134, or pSV1 (not shown), whereas the fluorescence was cytoplasmic with D10 (panel C). Co-microinjection of pTOT-425 and D10 resulted in both nuclear and cytoplasmic fluorescence in the absence of antagonists (panels D and E), whereas, in most of the cases, only the cytoplasm was fluorescent in the presence of Tamoxifen and RU486 (panels F and G). In contrast, both the cytoplasm and the nucleus were fluorescent when pTOT-134 and D10 were co-microinjected in the absence (panels H and I) or in the presence of the antagonists (panels J and K), supporting the conclusion that expression of pTOT-425, but not of pTOT-134, was dependent on the presence of steroid hormones.

Table II. Effect of oestradiol, progesterone and antagonists (Tamoxifen and RU486) on the activity of the ovalbumin promoter in primary cultures of chicken oviduct cells maintained in steroid-stripped medium (SM)

Recombinants	Immunofluorescent cells (% of pSV1)					
	Culture medium					
	SM	+ Oestradiol (10^{-8} M)	+ Progesterone (10^{-9} M)	+ Tamoxifen (10^{-6} M)	+ RU486 (2×10^{-6} M)	+ Tamoxifen + RU486
Control (pSV1)	100% (A)	100% (B)	100% (C)	100% (D)	100% (E)	100% (F)
pTOT-134	60%[42–100] (7)	74%[58–84](4)	75%[60–85](5)	75%[58–91](2)	n.d.	60%[52–71](4)
pTOT-295	60%[50–72] (3)	64%[44–83](2)	65%[49–81](2)	n.d.	n.d.	53%[42–65](2)
pTOT-425	26%[10–42](13)	60%[34–91](8)	55%[34–75](7)	12%[8–17](4)	13%[5–20](4)	4%[0–8](7)

The pTOT series of ovalbumin promoter recombinants were microinjected and the cells were processed as described in footnote to Table I. Hormones, antagonists, or both, were added immediately after the microinjection. The results were expressed as in Table I. 100% pSV1 controls corresponded to: (A)50%[43–60](15); (B) 49%[36–74](9); (C) 53%[34–68](9); (D) 50%[45–55](3); (E) 55%[45–67](4); (F) 48%[36–68](8). N.d.: not determined.

In the absence of steroid hormones, the activity of the ovalbumin promoter is blocked by sequences located upstream to −295

To demonstrate directly that the expression of pTOT-425 and pTOT-1348, but not of pTOT-56, pTOT-134 and pTOT-295, is dependent on the presence of steroid hormones, primary cultured oviduct cells were maintained in a stripped medium (SM) containing 10% FCS pretreated with dextran-charcoal to remove most, but not all, of the endogenous steroid hormones (Westley and Rochefort, 1980). The recombinants pTOT-56 (not shown), pTOT-134 and pTOT-295 were expressed similarly in SM and in MFCS (compare MFCS and SM in Tables I and II), whereas the expression of pTOT-425 and pTOT-1348 (not shown) was significantly lower in SM than in MFCS. The addition of Tamoxifen and/or RU486 to the SM culture medium resulted in a further decrease in expression of pTOT-425, whereas pTOT-134 and pTOT-295 were unaffected. It is important to note that phenol red, which is a weak oestrogen agonist (Berthois *et al.*, 1986), was present in all culture media.

These results, and those obtained in MFCS medium indicate that the −295 to −425 5′-flanking segment of the ovalbumin promoter contains sequences belonging to a negative regulatory element which mediates the 'repression' of pTOT-425 and pTOT-1348 in the absence of steroid hormones. As expected, and in agreement with the known effect of steroid hormones on ovalbumin gene transcription *in vivo* (see Introduction), this repression was relieved by the addition of either oestradiol, progesterone or glucocorticoid (Table II and results not shown). The addition of any of them to the SM medium resulted in an increase of pTOT-425 or pTOT-1348 (not shown) expression to a level similar to that observed for pTOT-134 and pTOT-295. An identical stimulation was obtained by adding oestradiol or progesterone to the SM medium containing RU486, thus confirming that this compound has no anti-progestin activity in oviduct tubular gland cells.

Unidirectionality of the negative regulatory element

Because many eukaryotic transcriptional regulatory elements function efficiently irrespective of their orientation (for reviews, see McKnight and Tjian, 1986; Wasylyk, 1986), we investigated whether the activity of the negative regulatory element present in the 5′-flanking region of the ovalbumin gene can function bidirectionally. Since the isolated −295 to −425 segment was not efficient at repressing the constitutive activity of the ovalbumin promoter elements present in pTOT-134 (our unpublished results), pTOT-425SAC and pTOT-425SACI were constructed. In pTOT-425SAC, a polylinker containing a *Sac*I site was added at position −134, and the −132 to −425 fragment was inserted further upstream. This fragment was inserted in the reverse orientation in pTOT-425SACI. A comparison of the results shown in Table III (see also Table I) indicates that the activity of pTOT-425SAC was nearly as sensitive as that of pTOT-425 to

Table III. The negative regulatory effect of the −132 to −425 region of the ovalbumin promoter is unidirectional

Medium	Immunofluorescent cells (% of pSV1)		
	Recombinants		
	pTOT-425	pTOT-425SAC	pTOT-425SACI
MFCS	48[35–70](5)	59[33–89](3)	52[39–66](6)
MFCS + Tamoxifen (10^{-6} M) RU486 (2×10^{-6} M)	14[10–19](6)	15[10–23](3)	44[30–56](6)

pTOT-425 (Figure 1A), pTOT-425SAC and pTOT-425SACI (Figure 1B) were microinjected in primary cultured chicken oviduct cells maintained in medium with foetal calf serum (MFCS) as described in footnote to Table I. Antagonists were added immediately after the microinjections. Results were expressed as in Table I. 45–65% of the cells microinjected with the control recombinant pSV1 were T-antigen positive.

Table IV. The ovalbumin sequence −132 to −425 represses the activity of the heterologous conalbumin promoter in the presence of the antagonists Tamoxifen and RU486

Medium	Immunofluorescent cells (% of pSV1)		
	Recombinants		
	pTOT-425	pTCT-102	pOVCON
MFCS	39[28–51](5)	56[43–68](3)	40[31–62](7)
MFCS + Tamoxifen (10^{-6} M) RU486 (2×10^{-6} M)	7[2–11](5)	49[34–58](3)	10[6–17](7)
+ Tamoxifen, RU486 Progesterone (10^{-9} M)	43[28–62](5)	50[47–53](3)	32[25–50](7)

pTCT-102 (Figure 1C and Dierich *et al.*, 1987), pTOT-425 (Figure 1A) and pOVCON (Figure 1C) were microinjected in primary cultured chicken oviduct cells maintained in medium with foetal calf serum (MFCS) as described in Table I. Antagonists and progesterone were added after the microinjection as indicated. The results were expressed as described in Table I. 53–75% of the cells microinjected with the control recombinant pSV1 were T-antigen positive.

Table V. Repression of the activity of the heterologous chicken β-globin promoter by the ovalbumin promoter sequence −132 to −425 in the presence of the antagonists Tamoxifen and RU486

Medium	Immunofluorescent cells (% of pSV1)					
	Recombinants					
	pTOT-425	pADG	pOVGLOB(115)	pOVLOBI(115)	pOVGLOB(59)	pOVGLOBI(59)
MFCS	50[36−56](5)	47[40−51](4)	50[39−70](5)	47[32−70](5)	43[36−49](5)	48[28−68](4)
MFCS + Tamoxifen (10^{-6} M) RU486 (2×10^{-6} M)	13[10−18](5)	48[38−68](3)	15[10−23](5)	44[35−59](5)	13[9−18](5)	48[26−78](4)
+ Tamoxifen, RU486 Progesterone (10^{-9} M)	56[42−65](5)	n.d.	42[27−56](5)	n.d.	41[20−55](5)	n.d.

The recombinants pOVGLOB(59), pOVGLOBI(59), pOVGLOB(115) and pOVGLOBI(115) (Figure 1D) were microinjected in primary cultured chicken oviduct cells maintained in medium with foetal calf serum (MFCS) as described in Table I. Antagonists and progesterone were added after the microinjections. The results were expressed as described in Table I. 54−78% of the cells microinjected with the control recombinant pSV1 were T-antigen positive. N.d.: not determined.

the effect of antagonist addition to the MFCS medium. The activity of pTOT-425SAC was also restored by the addition of progesterone in the presence of both antagonists (results not shown). In contrast, the expression of pTOT-425SACI was not decreased by the addition of the antagonists RU486 and Tamoxifen, suggesting that the negative regulatory element contained in the −132 to −425 fragment cannot function bidirectionally to repress the constitutive activity of the downstream ovalbumin promoter elements.

Repression of heterologous promoter elements

The results obtained with pTOT-425SAC, in which the natural continuity between the constitutive promoter elements of the ovalbumin gene and the sequence located further upstream is interrupted, prompted us to investigate whether the −132 to −425 fragment could function on heterologous promoters as a negative regulatory element whose effect could be relieved by steroid hormones. We tested first the recombinant pOVCON (Figure 1C), in which the ovalbumin gene −132 to −425 fragment is inserted upstream to the +62 to −102 conalbumin gene promoter fragment present in pTCT-102 (Moreau *et al.*, 1981; Dierich *et al.*, 1987). This recombinant is expressed selectively in primary cultured hepatocytes and oviduct tubular gland cells, but the conalbumin promoter activity is insensitive to the presence of steroid hormones in the culture medium (Dierich *et al.*, 1987 and Table IV). The introduction of the −132 to −425 ovalbumin fragment into pTCT-102 did not alter significantly its expression in the MFCS medium (compare pTCT-102 and pOVCON in Table IV). However, the activity of pOVCON was reduced drastically when the antagonists Tamoxifen and RU486 were added, and this repression was relieved by the addition of progesterone, which had no effect on pTCT-102 expression.

Since the conalbumin gene is expressed under steroid hormone control in mature oviduct *in vivo*, we constructed the next recombinants in which the ovalbumin −132 to −425 region was inserted upstream of the promoter elements of a gene whose activity is not controlled by steroid hormones and which is never expressed in the oviduct (Bellard *et al.*, 1977; Gariglio *et al.*, 1981 and refs therein). In pADG, the chicken β-globin promoter region from +43 to ~ −600 is inserted upstream to the SV40 T-antigen coding sequence (Figure 1D). The recombinant pOVGLOB(59) and pOVGLOB(115) were derived from pADG by inserting, in the 'transcription' sense, the ovalbumin −132 to −425 fragment at positions −59 and −115 of the β-globin promoter respectively. Note that the CAAT box upstream element of the β-globin gene promoter is not present in pOVGLOB(59) (Dolan *et al.*, 1983). In pOVGLOBI(59) and pOVGLOBI(115), the same ovalbumin promoter fragment was inserted at the same positions, but in the reverse orientation. The recombinant pADG is constitutively expressed in the primary cultured tubular gland cells (and also in oviduct fibroblasts − not shown), indicating that these cells contain some of the transcription factors required for the activity of the β-globin gene promoter, even though this gene is not expressed in the oviduct. The expression of both pOVGLOB(59) and pOVGLOB(115) was repressed by the addition of the antagonists Tamoxifen and RU486, and restored by the addition of progesterone to the culture medium (Table V). In contrast, the activity of pOVGLOBI(59) and pOVGLOBI(115) was insensitive to the addition of the antagonists. Since both pOVGLOB(59) and pOVGLOB(115) were similarly repressed by the ovalbumin negative regulatory element, it is unlikely that its inactivity in pOVGLOBI(59) and pOVGLOBI(115) could be due to an inappropriate stero-positioning with respect to the globin promoter elements. We therefore conclude that the negative control element present in the 5′-flanking region of the ovalbumin gene acts unidirectionally both on the ovalbumin constitutive promoter elements located downstream to −134 and on heterologous promoters.

Discussion

Cell-specific activity of constitutive and negative regulatory elements of the ovalbumin gene promoter

In the chicken, the ovalbumin gene promoter functions only in tubular gland cells of the oviduct after administration of steroid hormones (see Introduction and Dierich *et al.*, 1987). We have shown in the accompanying study (Dierich *et al.*, 1987) that all of the ovalbumin promoter recombinants of the pTOT series are inactive, when microinjected into a variety of heterologous non-chicken and chicken cells, including the fibroblasts present in primary cultures of oviduct. However, each of these recombinants is efficiently expressed in primary cultures of chicken embryonic hepatocytes, irrespective of the presence of steroid hormones, although the ovalbumin gene is not expressed in the liver of embryos treated with steroid hormones, in spite of the presence of the oestrogen receptor. In contrast, the pTOT recombinants are not expressed in primary cultured hepatocytes derived from adult liver (Dierich *et al.*, 1987). These results, and those presented here, lead to the following conclusions for the organization of the ovalbumin promoter and the regulation of its activity. (i) The

ovalbumin promoter contains steroid-independent constitutive elements which are located between −56 and −1. The activities of these elements require a cell-specific *trans*-acting factor(s) present in embryonic hepatocytes and oviduct tubular gland cells, but not in the adult liver and in the other chicken cells which have been tested. Thus the lack of expression of the ovalbumin gene in embryonic liver may be due to a developmentally regulated *cis*-acting negative mechanism which results in a permanent 'closing' of the gene (see Dierich *et al.*, 1987, for further discussion). We cannot exclude the possibility that additional constitutive elements could be present either upstream or downstream from the −56 to −1 region, although both pTOT-295 and a recombinant containing the −134 to +107 ovalbumin promoter region (our unpublished results) are not constitutively expressed in hepatocytes and oviduct cells at a level higher than pTOT-56. The effect of such possible constitutive elements may not have been detected in the present study, because the immunofluorescence assay only partially reflects the activity of the promoter (see Dierich *et al.*, 1987). For example, this may be the case for the CAAT box element which is located around −80 (Pastorcic *et al.*, 1986 and Sagami *et al.*, 1986). (ii) Some of the sequences located between −295 and −425 belong to a negative regulatory element(s) which mediate(s) the repression of the ovalbumin promoter region in oviduct tubular gland cells. Most probably, the activity of this negative regulatory element requires the presence of an oviduct tubular gland cell-specific *trans*-acting 'repressor', since pTOT-425 is not repressed in embryonic hepatocytes. (iii) This repression is relieved in the presence of either oestrogens, progestins, or glucocorticoids.

Thus, different mechanisms appear to be responsible for the inactivity of the ovalbumin gene promoter in different cell types. In most cases, the factors interacting with the constitutive element(s) are probably absent (as in the cases of fibroblasts, kidney cells and adult hepatocytes) and in addition, the gene has been permanently 'closed' during development by some epigenetic events. In a few cases, as in embryonic hepatocytes, the permanent 'closing' of the gene may be solely responsible for the inactivity of the promoter, since the factor(s) interacting with the constitutive element(s) appear(s) to be present. This 'closing' mechanism is presumably bypassed by microinjection of the recombinants. Finally, in oviduct tubular gland cells, the constitutive factor(s) is(are) present, but, in the absence of steroid hormones, the constitutive promoter activity appears to be repressed by a cell-specific *trans*-acting repressor interacting with a negative regulatory element. This regulation is markedly different from those which have been previously found for other glucocorticoid- or oestrogen-responsive promoters (see Introduction and below), where no negative regulatory elements have been found, and the effect of the hormone−receptor complex is to activate promoter elements whose intrinsic activity is very low. The negative regulation of the ovalbumin promoter region in cultured tubular gland cells in the absence of steroid hormones has not been observed by Dean *et al.* (1983, 1984). The reason for this discrepancy is unknown. It does not appear to be due to the presence of additional 5′-flanking sequences in their constructs, since we have observed the same negative regulation with a chimeric recombinant containing the −425 to +107 region of the ovalbumin promoter (data not shown). However, it may be related to the presence of the SV40 enhancer in their ovalbumin promoter chimeric recombinant, since we have noticed that the negative effect of the −295 to −425 sequence is not seen when this enhancer is inserted in recombinants pTOT-425 and pTOT-1348 (unpublished results). The 'masking' effect of an enhancer on the function of a negative control region has also been observed by Gonzalez and Nebert (1985)

Properties of the negative regulatory element and function of the ovalbumin gene promoter

The −132 to −425 control region of the ovalbumin promoter behaves as an independent functional unit containing the regulatory elements necessary for both repression (in the presence of antagonists) and induced derepression (in the presence of steroid hormones) of linked heterologous promoters. As for enhancer elements (Wasylyk *et al.*, 1983, 1984), these effects are not dependent on the presence of upstream promoter elements and seem to be, at least to some extent, 'distance'-independent [compare the results obtained with pOVGLOB(59) and (115) in Table V]. However, in contrast to most enhancers (but see Hen *et al.* 1983), the effect of the −132 to −425 ovalbumin control region appears to be undirectional. We cannot exclude at present that this unidirectionality could be artefactual and due to misalignments between the ovalbumin control region and the linked heterologous promoter elements, similar to those previously observed for enhancers (Takahashi *et al.*, 1986). This possibility is, however, unlikely, since the wild-type spacing has also been altered in the 'sense of transcription' recombinants pTOT-425SAC, pOVGLOB(59) and pOVGLOB(115), in which the ovalbumin control region is functional.

Evidence supporting the existence of *cis*-acting negative regulatory transcriptional elements in higher eukaryotes has been recently provided by several studies (Gonzalez and Nebert, 1985; Gorman *et al.*, 1985; Hen *et al.*, 1985; Jones *et al.*, 1985; Boss and Strominger, 1986; Goodbourn *et al.*, 1986; Jalinot and Kédinger, 1986; Nir *et al.*, 1986; Miyazaki *et al.*, 1986; Wasylyk and Wasylyk, 1986). However, the underlying molecular mechanisms are still poorly understood. In several cases (see Gonzalez and Nebert, 1985; Goodbourn *et al.*, 1986; Jalinot and Kédinger, 1986; Nir *et al.*, 1986) the negative regulatory region appears to block the activity of constitutive or inducible, adjacent or overlapping positive regulatory regions with enhancer-like properties. Our present results do not support the idea that the −132 to −425 ovalbumin promoter region could contain positive regulatory elements adjacent to, or intermingled with, the negative regulatory region, since the activity of the hormonally de-repressed recombinants containing the −132 to −425 sequence was not higher than that of the corresponding recombinants lacking this sequence. However, due to the quantitative limitation of the immunofluorescence assay (see above), we cannot exclude that the effect of positive regulatory elements may have been unnoticed in the present study. This could explain why we have not seen any significant difference in the levels of expression of pTOT-295 and pTOT-134 in the presence or absence of steroid hormone receptors, whereas Dean *et al.* (1983, 1984) have reported that sequences located between −197 and −143 are responsible for a 5- to 10-fold induction of the ovalbumin promoter by either progesterone or oestradiol. The nature of the repressor and the exact location of its binding sequences are unknown. Recent studies have shown that steroid receptors are permanently located in the nucleus (Sheridan *et al.*, 1979; Welshons *et al.*, 1984; King and Greene, 1984), which suggests that unfilled receptors could act as repressors. Such a possibility is, however, not supported by the lack of repression in embryonic hepatocytes which contain oestrogen receptor molecules, although perhaps at too low a level to be efficient (Lazier, 1978). Furthermore, our preliminary results from an internal deletion study indicate that sequences located in the −326 to −300 region are

required for efficient repression, which is far upstream from the −197 to −143 region which may bind the oestrogen and progesterone receptors (see above and Weisz *et al.*, 1986). The situation may in fact be even more complex than anticipated, as indicated by the observation (our unpublished data) that a −132 to −224 internal deletion in pTOT-425 results in constitutive expression in the absence of hormones, whereas repression, but not steroid hormone-induced derepression, is observed with a −189 to −224 internal deletion. Thus, two negative regulatory regions may be separated by a region binding the steroid hormone receptors. It is tempting to speculate that the binding of the steroid hormone−receptor complexes may disrupt interaction between *trans*-acting repressors bound to the negative regulatory regions, thereby releasing the repression exerted on the constitutive elements located downstream. According to this view, the repressing effect could be mediated by protein−protein interactions similar to those which have been postulated for explaining the properties of enhancer elements (Ptashne, 1986; Takahashi *et al.*, 1986). Finally, additional regulatory elements modulating the activity of the ovalbumin promoter, and not revealed using the present microinjection assay, may be present upstream from the 'blocker' sequences, since nuclease sensitivity studies on oviduct chomatin have revealed the existence of steroid hormone-inducible hypersensitive regions centered at approximately 0.8, 3.2 and 6.0 kb upstream from the ovalbumin cap site (Kaye *et al.*, 1986). The situation may be analogous to that of the mouse P_1450 gene whose promoter is controlled by an inducer−receptor complex similar to steroid hormone−receptor complexes (Gonzalez and Nebert, 1985; Jones *et al.*, 1985). In this case, a negative regulatory region located between −389 and −823 is indeed preceded by a enhancer-like ligand-inducible positive regulatory element. Studies are in progress to establish a more quantitative and more convenient assay than nuclear microinjection to further dissect the various components of the ovalbumin promoter.

Materials and methods

DNA recombinants

The pTOT ovalbumin promoter−T-antigen chimeric recombinant series (Figure 1A) is described in the accompanying paper (Dierich *et al.*, 1987). pTOT-425SAC or pTOT-425SACI were constructed as follows (see Figure 1B). The ovalbumin fragments *Rsa*I−*Ava*II (−425 to −134) and *Ava*II−*Rsa*I (−134 to −1) were subcloned to the *Sma*I site of M13mp10 and M13mp11 respectively. The fragments *Sac*I−*Bam*HI (containing the ovalbumin region −134 to −1) and *Sal*I−*Sac*I (containing the ovalbumin region −425 to −132) or *Sac*I−*Sal*I (containing the ovalbumin region −425 to −132) were excised and purified from the M13 subclones, and then ligated into pEMP digested by *Sal*I and *Bam*HI to yield pTOT-425SAC and pTOT-425SACI. pOVCON (Figure 1C) was obtained by partial *Bam*HI digestion of the conalbumin recombinant pTCT-102 (Dierich *et al.*, 1987) and insertion of the ovalbumin promoter fragment from −132 to −425 at position −102 of the conalbumin promoter. pOVGLOB(115) and pOVGLOB(59) and the reverse orientation pOVGLOBI(115) and pOVGLOBI(59) were obtained as follows (see Figure 1D): the ovalbumin fragment −134 to −425 subcloned in M13 was excised by *Eco*RI−*Xba*I digestion and inserted in either orientation at position −59 [pOVGLOB(59) and pOVGLOBI(59)] and −115 (*Sma*I site) [pOVGLOB(115) and pOVGLOBI(115)] of the plasmid pADG (Dierich *et al.*, 1987) using *Eco*RI linkers. Orientation of the ovalbumin sequence with respect to the globin sense of the transcription was determined by enzyme restriction mapping.

All other materials and methods were as described in the accompanying paper (Dierich *et al.*, 1987).

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References

Beato,M., Scheidereit,C., Krauter,P., von der Ahe,D., Janich,S., Cato,A.C.B., Suske,G. and Westphal,H.M. (1985) In Reeck,G.R., Goodwin,G.H. and Puigdomenech,P. (eds), *Chromosomal Proteins and Gene Expression*. Plenum, NY, p. 121−143.

Bellard,M., Gannon,F. and Chambon,P. (1977) *Cold Spring Harbor Symp. Quant. Biol.*, **42**, 779−791.

Benoist,C. and Chambon,P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3865−3869.

Berthois,Y., Katzenellenbogen,J.A. and Katzenellenbogen,B.S. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2496−2500.

Boss,J.M. and Strominger,J.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9139−9143.

Breathnach,R., Mantei,N. and Chambon,P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 740−744.

Brown,A.M.C., Jeltsch,J.M., Roberts,M. and Chambon,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6344−6348.

Chambon,P., Gaub,M.P., LePennec,J.P., Dierich,A. and Astinotti,D. (1984) In Labrie,F. and Proulx,L. (eds), *Proc. Int. Congr. Endocr.*, Elsevier Science Publ., North Holland, **7**, pp. 3−10.

Dean,D.C., Knoll,B.J., Riser,M.E. and O'Malley,B.W. (1983) *Nature*, **305**, 551−554.

Dean,D.L., Gope,R., Knoll,B.H., Riser,M.E. and O'Malley,B.W. (1984) *J. Biol. Chem.*, **259**, 9967−9970.

Dean,D.C., Gope,R., Knoll,B.H., Riser,M.E. and O'Malley,B.W. (1984) *J. Biol. Chem.*, **259**, 9967−9970.

Dierich,A., Gaub,M.P., LePennec,J.P., Touitou,I. and Chambon,P. (1987) *EMBO J.*, **6**, 2305−2312.

Dolan,M., Dogdson,J.B. and Engel,J.D. (1983) *J. Biol. Chem.*, **258**, 3983−3990.

Druege,P.M., Klein-Hitpass,L., Green,S., Stack,G., Chambon,P. and Ryffel,G.U. (1986) *Nucleic Acids Res.*, **14**, 9329−9337.

Gariglio,P., Bellard,M. and Chambon,P. (1981) *Nucleic Acids Res.*, **9**, 2589−2598.

Gaub,M.P., Dierich,A., LePennec,J.P., Astinotti,D., Touitou,I. and Chambon,P. (1985) *Cold Spring Harbor Symp. Eucaryotic Transcription, Current communications*, 123−131.

Goodbourn,S., Burstein,H. and Maniatis,T. (1986) *Cell*, **45**, 601−610.

Gorman,C., Rigby,P.W. and Lane,D.P. (1985) *Cell*, **42**, 519−526.

Groyer,A., Leboul,Y., Joab,I., Radanyl,C., Renoir,J.M., Robel,P. and Baulieu,E.E. (1985) *Eur. J. Biochem.*, **149**, 445−451.

Hen,R., Borrelli,E., Sassone-Corsi,P. and Chambon,P. (1983) *Nucleic Acids Res.*, **11**, 8747−8760.

Hen,R., Borrelli,E. and Chambon,P. (1985) *Science*, **230**, 1391−1394.

Jalinot,P. and Kédinger,C. (1986) *Nucleic Acids Res.*, **14**, 2651−2669.

Jones,P.B.C., Galeazzi,D.R., Fischer,J.M. and Whitlock,J.P.,Jr (1985) *Science*, **227**, 1499−1502.

Jost,J.P., Geiser,M. and Seldran,M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 988−991.

Jung-Testas,I., Garcia,T. and Baulieu,E.E. (1986) *J. Steroid Biochem.*, **24**, 273−279.

Kalderon,D., Richardson,W.D., Markham,A.F. and Smith,A.E. (1984) *Nature*, **311**, 33−37.

Kaye,J.S., Pratt-Kaye,S., Bellard,M., Dretzen,G., Bellard,F. and Chambon,P. (1986) *EMBO J.*, **5**, 277−285.

King,W.J. and Greene,G.L. (1984) *Nature*, **307**, 745−747.

Klein-Hitpass,L., Schorpp,M., Wagner,U. and Ryffel,G.U. (1986) *Cell*, **46**, 1053−1061.

Lazier,C.B. (1978) *Biochem. J.*, **174**, 143−152.

Lemeur,M., Glanville,N., Mandel,J.L., Gerlinger,P., Palmiter,R. and Chambon,P. (1981) *Cell*, **23**, 561−571.

McKnight,S.L. and Tjian,R. (1986) *Cell*, **46**, 795−805.

Miyazaki,J.I., Appella,E. and Ozato,K. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9537−9541.

Moreau,P., Hen,R., Wasylyk,B., Everett,R., Gaub,M.P. and Chambon,P. (1981) *Nucleic Acids Res.*, **9**, 6047−6068.

Nir,U., Walker,M.D. and Rutter,W.J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3180−3184.

Pastorcic,M., Wang,H., Elbrecht,A., Tsai,S.Y., Tsai,M.J. and O'Malley,B.W. (1986) *Mol. Cell. Biol.*, **6**, 2784−2791.

Philibert,D., Derardt,R. and Teusch,G. (1981) VIII Int. Congr. of Pharmacology, Tokyo, Abstract no. 1463.

Ptashne,M. (1986) *Nature*, **322**, 697−701.

Renkawitz,R., Schütz,G., von der Ahe,D. and Beato,M. (1984) *Cell*, **37**, 503–510.
Sagami,I., Tsai,S.Y., Wang,H., Tsai,M.J. and O'Malley,B.W. (1986) *Mol. Cell. Biol.*, **6**, 4259–4267.
Seiler-Tuyns,A., Walker,P., Martinez,E., Merillat,A.M., Givel,F. and Wahli,W. (1986) *Nucleic Acids Res.*, **14**, 8755–8770.
Sheridan,P.J., Anselmo,V.L., Buchanan,J.M. and Martin,P.M. (1979) *Nature*, **282**, 579–582.
Takahashi,K., Vigneron,M., Matthes,H., Wildeman,A., Zenke,M. and Chambon,P. (1986) *Nature*, **319**, 121–126.
Wasylyk,B., Wasylyk,C., Augereau,P. and Chambon,P. (1983) *Cell*, **32**, 503–514.
Wasylyk,B., Wasylyk,C. and Chambon,P. (1984) *Nucleic Acids Res.*, **12**, 5589–5608.
Wasylyk,C. and Wasylyk,B. (1986) *EMBO J.*, **5**, 553–560.
Wasylyk,B. (1986) In Reznikoff,W. and Gold,L. (eds), *Maximizing Gene Expression*. Butterworths, pp. 79–99.
Weisz,A., Coppola,L. and Bresciani,F. (1986) *Biochem. Biophys. Res. Commun.*, **139**, 396–402.
Welshons,W.K., Liebermann,M.E. and Gorski,J. (1984) *Nature*, **307**, 747–749.
Westley,B. and Rochefort,H. (1980) *Cell*, **20**, 353–362.
Yamamoto,K.R. (1986) *Molecular Developmental Biology*. Alan R.Liss Inc., NY, 131–148.

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