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Positive and Negative Regulatory Elements Control the Steroid-Responsive Ovalbumin Promoter†

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ABSTRACT: Steroid hormones regulate the transcriptional activity of the chicken ovalbumin gene both in vivo and in cell culture. To identify the regulatory elements involved, primary oviduct cell cultures were transfected with constructs containing the promoter and 5′-flanking region of the ovalbumin gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. Induction of the OvCAT genes by estrogen, progesterone, or corticosterone mimics that of the endogenous ovalbumin gene, indicating that the transfected DNA is accurately regulated. Deletion analysis revealed that a steroid response element (SRE) resides between nucleotide coordinates −880 and −585 and that a negative regulatory element (NRE) resides between −350 and −248 in the ovalbumin gene. Thus, an NRE represses expression of the ovalbumin gene unless steroid hormones relieve this negative control through interactions involving a more distal SRE. Neither the SRE nor the NRE alone regulates the heterologous thymidine kinase promoter, suggesting either that they function as a single entity or that they are conditional regulatory elements. The NRE is functional in MCF-7 cells, but the SRE cannot be activated by steroids in this heterologous estrogen-responsive cell line. These data indicate that the steroid–receptor complex induces the ovalbumin gene through direct or indirect actions at an SRE to relieve repression at an NRE.

For 2 decades, the chicken oviduct has served as a model to study the regulation of eucaryotic gene expression, in part because few gene regulatory molecules other than steroid receptors have been identified in higher eucaryotes. As a result, the biology of this system is well-defined [for a review, see Sanders and McKnight (1986)]. In sexually immature birds, estrogen initiates differentiation of the tubular gland cells of the oviduct and induces the mRNAs for the major egg white proteins, ovalbumin, transferrin or conalbumin, lysozyme, and ovomucoid, by enhancing both the transcription rates of these genes and the stability of the resultant mRNAs. In vitro experiments revealed that estrogen requires the permissive effects of insulin (Evans & McKnight, 1984) and corticosterone (Sanders & McKnight, 1986) to exert its effects on transcription. After primary exposure to estrogen in vivo, three other classes of steroids, the androgens, glucocorticoids, and progestins, can also induce the egg white genes, making this model unique for investigations of how the specificity of induction of target genes by different steroid hormones is achieved.

Relatively little is known about the regulatory elements controlling the ovalbumin gene. While the canonical TATA and CAAT boxes typical of highly active promoters have been characterized (Zarucki-Schulz et al., 1982), the sequences required for regulation by estrogen or other steroid hormones have not been defined. Competitive filter binding assays from two laboratories (Mulvihill et al., 1982; Compton et al., 1983) have identified areas in the 5′-flanking region that effectively compete with nonspecific DNA for partially purified progesterone receptor, but the strongest regions identified by each group do not overlap. Likewise, ovalbumin fusion genes were transfected into cultured oviduct cells, but no consensus was reached about the sequences required for regulation by estrogen or progesterone (Dean et al., 1983, 1984; Chambon et al., 1984; Gaub et al., 1987). These discrepancies may result from differences in receptor preparations, cell culture conditions, fusion genes, and assay systems.

To define the sequences in the ovalbumin gene required for regulation by estrogen, we have developed a primary tubular gland cell culture system for chicken oviduct in which the endogenous ovalbumin gene is induced by hormones to the same extent that it is induced in vivo (Sanders & McKnight, 1985). As described in this paper, a transfection protocol has been adapted to these cells that allows the assay of promoter fusion genes without disturbing the responsiveness of the en-

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dogenous ovalbumin gene to hormones. Deletional analysis has been used to define both positive and negative regulatory elements modulating the ovalbumin promoter.

Experimental Procedures

Culture and Transfection of Tubular Gland Cells. Tubular gland cells were isolated as previously described (Sanders & McKnight, 1985) using enzymatic dissociation. After isolation, approximately 5×10^6 cells were placed in 100-mm tissue culture plates containing 10 mL of transfection medium [Dulbecco's modified Eagle's medium mixed 1:1 with Ham's Nutrient F12, 5% charcoal-stripped horse serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and fungizone (500 ng/mL)]. The DNA–calcium phosphate solutions containing 15 μg/mL DNA, except as indicated below, were prepared as described by van der Eb and Graham (1980) although the coprecipitates were formed on ice rather than at room temperature. In the experiments depicted by Figure 5, equal molar concentrations of DNA were transfected such that the molarity of pOvCAT-1.3 was equal to the 15 μg/mL normally used with the transfections. Two milliliters of the DNA–calcium phosphate solution was pipetted gently onto the cells, and they were incubated at 41 °C for 4 h. To remove the DNA, the cells plus medium were removed from the plates by pipet into 50-mL conical tubes and the cells pelleted by centrifugation. Cells were resuspended in transfection medium and centrifuged again. The cells were resuspended in a small volume of culture medium (DME:F12, 0.1% BSA, penicillin, streptomycin, and fungizone as above) containing no hormones, and all cells transfected with a particular DNA were pooled. The cells were then aliquoted into tissue culture dishes containing the culture medium plus hormones (estrogen, 1×10^{-7} M; corticosterone, 1×10^{-6} M; insulin, 50 ng/mL, or progesterone, 1×10^{-8} M) as indicated and cultured at 41 °C in serum-free medium as described (Sanders & McKnight, 1985).

As discussed briefly under Results, the transfection efficiency of primary oviduct cells is very low (about 1 in 500 cells), making it virtually impossible to confirm our results by analysis at the mRNA level. Instead, a number of additional controls have been employed to ensure the accuracy of our results. All cells transfected with a particular plasmid were pooled after the transfection and were then aliquoted into dishes with media containing the appropriate hormones to ensure that there were no differences in transfection efficiency. The transfections were repeated several times with different DNA preparations. In addition, to confirm that tubular gland cells were transfected rather than other cell types, dual antibody staining was performed. A fluorescein-labeled second antibody against T-antigen and a rhodamine-labeled second antibody against ovalbumin showed that the cells that expressed the transfected SV40 T-antigen gene also made ovalbumin.

CAT Assay. CAT activity was measured in sonicated cell extracts prepared 24–48 h after transfection essentially as described by Gorman et al. (1982) and as modified by Mercola et al. (1985). All reactions were incubated at 37 °C and contained 0.3 μCi of [^{14}C]chloramphenicol (60 mCi/mmol) and 150 μg of protein as determined by the Bradford assay (1976) using bovine γ-globulin as a standard. To increase the sensitivity of the CAT assay, the reactions were incubated for 16 h, with 4.4 mM acetyl coenzyme A to maintain linearity. For quantitation, radioactive spots on the chromatogram were cut out and counted; 100% corresponds to about 450 000 cpm. The background, about 0.01%, as determined from a reaction without added cell extract, has been subtracted.

Solution Hybridization. Total cellular RNA was isolated and hybridized in solution to an ovalbumin cDNA probe as previously described (Sanders & McKnight, 1985).

Plasmids. pMMTVCAT (Cato et al., 1986), pTKCAT (Miksicek et al., 1986), pLysCAT, and pLysTKCAT were kindly provided by Richard Miksicek and Gunter Schutz.

pOvCAT-1.3 was constructed from a genomic ovalbumin subclone (provided by P. Chambon) as described below. The 1.7-kb *Pst*4–*Eco*6 fragment from the ovalbumin gene (Gannon et al., 1979) was partially digested with *Alu*I to generate a fragment terminating at +7, the first *Alu*I site in the first exon. By blunt-ending with Klenow and ligating on *Cla*I linkers, the *Alu*I site was changed to a *Cla*I site. Similarly, the *Pst*4 site was cleaved, blunt-ended, and religated in the presence of *Eco*RI linkers. pTkCAT was digested with *Bgl*II and *Bam*HI to release the thymidine kinase promoter, and the vector was blunt-ended using Klenow. The *Eco*RI–*Cla*I fragment was blunt-ended with Klenow and ligated into the CAT vector.

pOvCAT-8 was constructed by ligating a 7.9-kb *Sal*I (−7400) to *Sph*I (−518) fragment from the ovalbumin gene obtained from P. Chambon (Royal et al., 1979) into pOvCAT-1.3 that had been digested with *Sal*I and *Sph*I. This generated an additional 7.1 kb of contiguous ovalbumin 5′-flanking region that was not on the pOvCAT-1.3 fusion gene.

To create the deletion mutants between −8000 and −350, standard restriction enzyme digests were performed, followed by filling in with Klenow and by blunt-end ligations. To make pOvCAT-7.4, pOvCAT-8 was digested with *Bam*HI and *Kpn*I to release a 1-kb fragment. pOvCAT-5.7 and pOvCAT-3.5 were made through a partial *Xba*I digest, removing 2.7 and 4.9 kb, respectively. A partial *Hin*dIII digest liberated a 4.4- or 5.6-kb fragment to make pOvCAT-4 or pOvCAT-2.8, respectively. pOvCAT-.88 was created by a partial *Xmn*I and complete *Pst*I digest of pOvCAT-1.3 to delete a 448 bp fragment. pOvCAT-.5 was made by digesting pOvCAT-1.3 with *Sph*I to remove an 800 bp piece. pOvCAT-.35, -.1, and -.08 were made by exonuclease III digestion of pOvCAT-1.3 (Henikoff, 1984).

To construct the reconstituted OvCAT fusion genes, two *Ssp*I fragments, one from −584 to −248 and the other from −1154 to −585, were blunt-ended and ligated into a *Hin*dIII site in the polycloning region that had been blunt-ended and was upstream of the ovalbumin sequences.

All plasmids were treated with proteinase K, CsCl purified, ribonuclease A treated, and run over a Bio-Gel A-5 column and were at least 50% supercoiled as judged by agarose gel electrophoresis.

Results

Primary Oviduct Cells Retain Hormone Responsiveness after Transfection. The transfer of mutated genes into responsive cell cultures is essential to establish the functional relevance of particular DNA sequences. Unfortunately, primary cell cultures are transfected only poorly and often do not retain their responsiveness in culture. We have developed techniques that allow reproducible gene transfer into primary cultures of oviduct cells without disrupting the induction of the endogenous ovalbumin gene. Although many gene transfer techniques including microinjection, DEAE-dextran, and protoplast fusion were tested (data not shown), calcium phosphate mediated DNA transfer proved to be the most effective at allowing maximal expression of both the endogenous and exogenous ovalbumin genes.

The time of transfection after cell isolation proved to be the most critical parameter for maintaining responsiveness to hormones (Figure 1). Oviduct cells were isolated as described

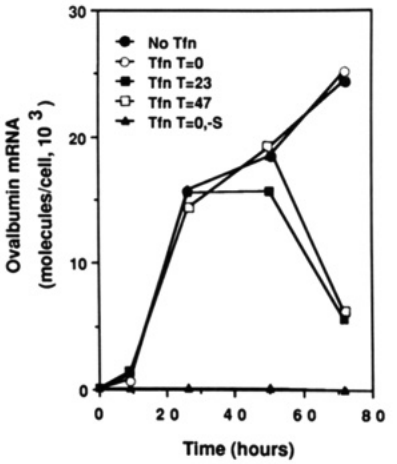

FIGURE 1: Tubular gland cells must be transfected immediately after isolation to retain responsiveness to steroids. Oviduct tubular gland cells were isolated (Sanders & McKnight, 1985) and divided into four groups: cultured but not transfected (closed circles): transfected immediately after isolation with an irrelevant plasmid and then cultured (open circles); transfected after 24 h in culture (closed squares); transfected after 48 h in culture (open squares). The cells were transfected by using the DNA–calcium phosphate coprecipitation method at the indicated times and were cultured in medium containing insulin (50 ng/mL) alone (–S) or insulin plus estrogen (1×10^{-7} M) and corticosterone (1×10^{-6} M; +S). At the indicated times, the cells were harvested, and the amount of endogenous ovalbumin mRNA was determined by solution hybridization using an ovalbumin cDNA probe. Each point was done in duplicate, and the experiment was done at least 3 times.

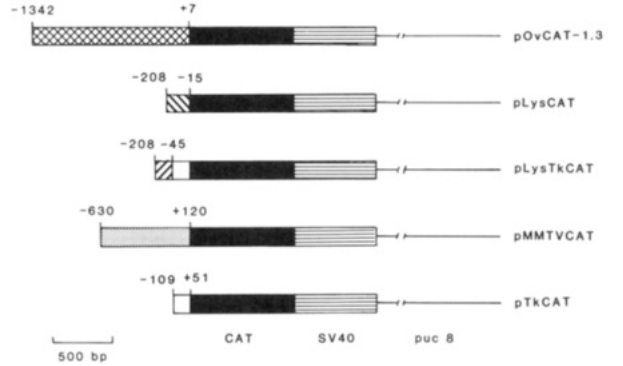

FIGURE 2: Steroid-regulated fusion genes. The promoters and/or 5′-flanking regions of several steroid-responsive promoters were fused to the CAT structural gene and are diagrammed: ovalbumin sequences are indicated by the cross-hatched bar, lysozyme sequences by hatched bars, TK sequences by white bars, MMTV sequences by dotted bars, CAT sequences by black bars, SV40 sequences by horizontal-lined bars, and plasmid sequences by a solid line.

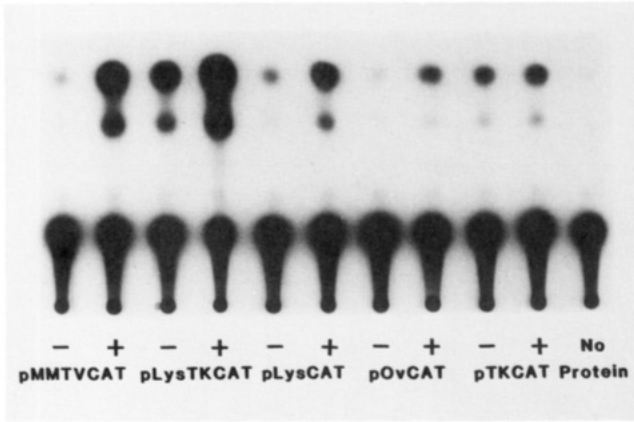

FIGURE 3: Steroid hormones regulate several steroid-responsive promoters in tubular gland cells. Tubular gland cells were transfected with the indicated plasmids at zero time and were then cultured with insulin alone (–S) or with insulin, estrogen, and corticosterone (+S). After 48 h, cell extracts were prepared by sonication and assayed for CAT activity in a 16-h reaction using 150 μg of protein, 4.4 mM acetyl coenzyme A, and 0.3 μCi of [^{14}C]chloramphenicol. Assay background was assessed by setting up a reaction without protein. The thin-layer chromatogram was exposed to film for 17 h. Two paltes of cells were transfected for each plasmid, and the experiment was repeated at least 3 times.

(Sanders & McKnight, 1985) and subjected to one of four treatments: the cells were (1) cultured without additional manipulation, (2) immediately transfected with DNA as described under Experimental Procedures followed by culture for 1 or 2 days, (3) cultured for 1 day prior to transfection, transfected, and then cultured for 2 days, or (4) cultured for 2 days, transfected, and then cultured for 1 more day. In all four cases, the cells were cultured with or without steroids. No differences were observed among the treatment groups when the cells were cultured with insulin alone (data not shown). When the cells were transfected immediately after isolation, the increase in endogenous ovalbumin mRNA in response to insulin plus steroids was indistinguishable from that of untransfected cells. In contrast, when the cells were cultured for 24–48 h prior to transfection, the accumulation of endogenous ovalbumin mRNA ceased after transfection, and the level of ovalbumin mRNA began to decline. This indicates that the transfection procedure itself disrupts the responsiveness of the cells when used at the later time points.

To determine the transfection efficiency over time in culture, tubular gland cells were transfected at 0, 24, or 48 h after isolation as described above using a plasmid containing the Simian virus 40 (SV40) T-antigen gene. Indirect immunofluorescence staining for nuclear T-antigen revealed that the transfection efficiency increased by 2–3-fold when the cells were transfected at 24 h rather than at zero time and by 6–8-fold when the cells were transfected at 48 h. However, when OvCAT fusion genes were transfected at 24 or 48 h, no steroid-dependent expression was observed (data not shown). Therefore, to obtain hormone-dependent expression of the endogenous ovalbumin gene and the transfected OvCAT genes, oviduct cells must be transfected immediately after isolation, even though the transfection efficiency is lowest at that time.

Steroid-Responsive Promoters Are Inducible in Oviduct Cell Cultures. Constructs containing the mouse mammary tumor virus (MMTV), lysozyme, ovalbumin, or thymidine kinase (TK) promoters and flanking regions fused to the CAT structural gene (Figure 2) were transfected into tubular gland cells immediately after their isolation (zero time), and the cells were cultured with insulin (–S) alone or with insulin, estrogen, and corticosterone (+S). The cells were harvested at 48 h, and the amount of CAT activity was determined. As shown in Figure 3, the combination of estrogen and a glucocorticoid induced the appropriate fusion genes, indicating that the cultured oviduct cells are capable of regulating exogenous, steroid-dependent genes.

The expression of the viral CAT fusion genes such as TK (Figure 3), SV40, and Rous sarcoma viruses (data not shown) was often slightly increased (2–4-fold) in oviduct cells exposed to steroids. This may represent a nonspecific effect of steroids on total transcriptional rates, mRNA stability, or translational activity. Estrogen has been shown to produce a 2–3-fold increase in the rate of initiation of total protein synthesis and a 40% increase in the rate of protein elongation in the oviduct (Palmiter, 1972). Because the increase in expression of the OvCAT, MMTVCAT, and LysCAT fusion genes is much greater than 10-fold, we believe steroids are specifically altering

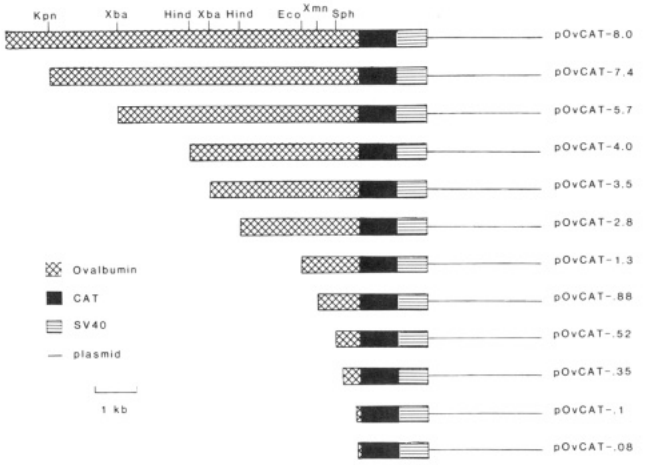

FIGURE 4: Deletions in the 5′-flanking region of pOvCAT. Deletions in the ovalbumin 5′-flanking region were created as described under Experimental Procedures using appropriate restriction enzyme digests or exonuclease III. The ovalbumin sequences remaining in the plasmids are depicted by the cross-hatched bars, and the numbers after the hyphen in each name indicate the length of the remaining ovalbumin sequence in kilobases. The CAT sequences are represented by the solid bars, the SV40 sequences by the horizontal-lined bars, and the plasmid sequences by the solid line.

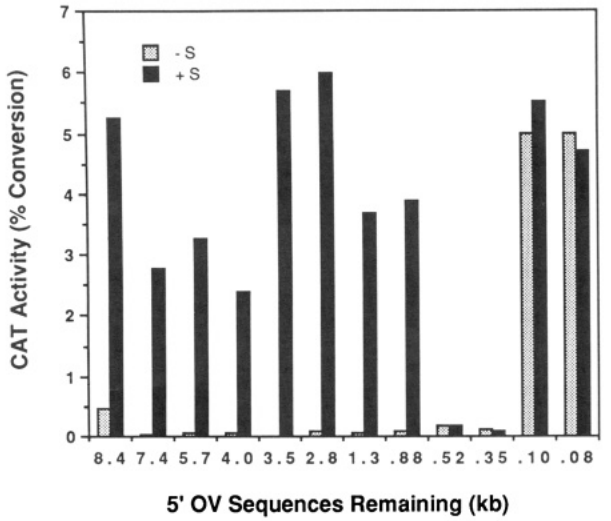

FIGURE 5: Two control elements, a steroid response element (−880 to −520) and a negative regulatory element (−350 to −100), regulate expression of the ovalbumin gene. Tubular gland cells were transfected with equal molar amounts (pOvCAT-1.3 was used at 15 μg of DNA/mL and the others were adjusted accordingly) of the OvCAT deletion mutants at zero time and were then cultured with insulin alone (stippled bars) or with insulin plus steroids (solid bars). After 24 h, the cells were harvested, and CAT activity was determined. Two plates of cells were transfected for each plasmid, and the experimental was repeated at least 3 times.

the transcriptional activity of these promoters. The data summarized in Figure 3 indicate that four steroid-responsive fusion genes, including pOvCAT-1.3, respond dramatically to steroid hormones in primary tubular gland cell cultures.

Ovalbumin Promoter Contains a Steroid Response Element and a Negative Regulatory Element. To determine where steroid response elements (SREs) reside in the ovalbumin gene, a fusion gene was prepared that contained 8 kb of 5′-flanking sequence (pOvCAT-8). A series of deletions were then made in the ovalbumin flanking region using appropriate restriction enzymes (Figure 4). The numbers after the hyphen indicate the length of ovalbumin DNA in kilobases remaining in the constructs. Because the sizes of the fusion genes differ significantly, equal molar concentrations of DNA were transfected as described under Experimental Procedures. After transfection at zero time, the cells were cultured for 2 days with insulin alone (−S) or with insulin and steroids (+S); Figure 5 shows the results of typical CAT assays with the serial deletions. Deletions between −8000 and −880 produced no significant difference in the capacity of the fusion genes to respond to steroids. However, when the sequences between −880 and −520 were deleted, all response to steroids was lost, and expression was minimal. Deleting the ovalbumin sequence to −350 produced no additional changes in expression. These data indicate that a SRE resides between −880 and −520 in the ovalbumin gene.

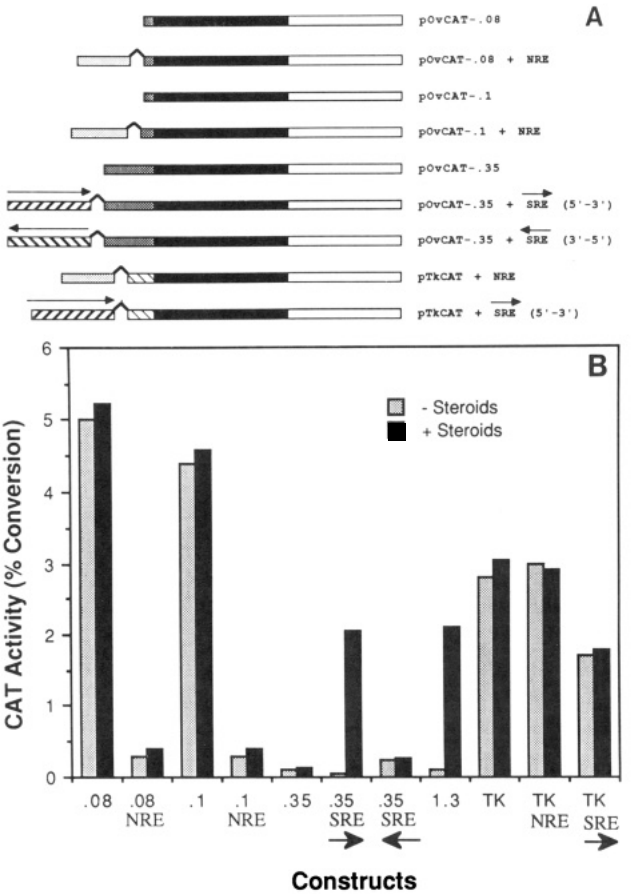

FIGURE 6: Reconstitution of the regulatory elements in the ovalbumin promoter. A fragment of DNA containing the negative regulatory element (−584 to −248) or the steroid response element (−1154 to −585) was ligated in the 5′ to 3′ orientation or in both orientations, respectively, to OvCAT deletion mutants lacking the corresponding function. Ovalbumin sequences are depicted by the hatched bars, CAT by the solid bars, and plasmid by the open bars. (B) Tubular gland cells were transfected at zero time with the indicated plasmids and were cultured with insulin alone (stippled bars) or with insulin plus steroids (solid bars). After 24 h, the cells were harvested, and CAT activity was determined. Two plates of cells were transfected with each plasmid, and the experiment was repeated at least 3 times.

More surprisingly, when the sequences between −350 and −100 were removed (pOvCAT-.1 and pOvCAT-.08), basal expression increased to levels seen with induced genes containing a SRE. However, the pOvCAT-.1 and -.08 fusion genes were no longer regulated by steroids. These data implicate a negative regulatory element (NRE) between −350 and −100 in the ovalbumin gene.

Through deletion analysis of the ovalbumin gene, two regulatory elements, a SRE and a NRE, have been identified. To demonstrate that the regions of DNA defined by these deletions can actually confer these specific activities, the regulatory regions were fused to homologous promoters lacking

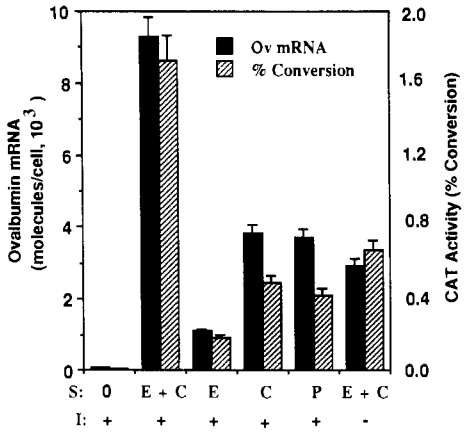

FIGURE 7: Expression of transfected OvCAT genes mimics that of the endogenous ovalbumin gene under various hormonal treatments. Tubular gland cells were transfected with pOvCAT-1.3 and were cultured with combinations of insulin, estradiol, corticosterone, or progesterone (1×10^{-8} Me) as indicated. After 24 h, the cells were harvested, and replicate dishes were assayed for the amount of endogenous ovalbumin mRNA (solid bars) or for CAT activity (hatched bars). Two plates of cells were transfected for each condition, and the experiment has been repeated at least 4 times.

the corresponding elements. As diagrammed in Figure 6A, a fragment of DNA (−584 to −248) containing the NRE was ligated in the correct orientation (5′ to 3′) upstream of the ovalbumin sequences in pOvCAT-.08 or pOvCAT-.1 as described under Experimental Procedures. Likewise, a fragment of DNA (−1154 to −585) containing the SRE was ligated into pOvCAT-.35 in both orientations. These fusion genes were transfected into tubular gland cells, the cells were cultured for 2 days with hormones as indicated, and cell extracts were assayed for CAT activity. As shown in Figure 6B, the NRE reduced expression of the pOvCAT-.08 and -.1 constructs over 10-fold when placed upstream of the ovalbumin promoter, and this repression was unaffected by steroids. These results in conjunction with those from Figure 5 suggest that the NRE resides between −350 and −248 and that the sequences between −247 and −100 are not an essential part of the NRE.

When a fragment of DNA containing the SRE (−1154 to −585) was placed in the 5′ to 3′ orientation with respect to the ovalbumin sequences in pOvCAT-.35, steroids increased its expression to the same extent that pOvCAT-1.3 was induced. These data and those from Figure 5 suggest that the SRE lies between −880 and −585. When the SRE was inserted 3′ to 5′, steroids did not induce expression. These preliminary results suggest that the SRE may not act like a typical enhancer in this system, although we cannot distinguish between a distance effect (the SRE is approximately 270 bp further upstream in the inverted position) or an orientation dependence.

To determine whether the SRE and NRE could confer their respective activities on a heterologous promoter, each was placed upstream of the TK promoter (Figure 6A). These fusion genes were transfected into cultured oviduct cells and the cells cultured in the presence and absence of steroids. The NRE did not repress expression of the TK promoter nor did the SRE confer steroid-dependent expression (Figure 6B). This suggests that these regulatory elements are either conditional and only act on their homologous promoter or that these two elements act as a single functional unit.

OvCAT Fusion Genes Mimic the Multihormonal Responses of the Endogenous Ovalbumin Gene. The ability of oviduct cells to regulate the expression of pOvCAT-1.3, which contains both the SRE and the NRE, in a manner analogous to that of the endogenous ovalbumin gene was examined under various

Table I: Expression of Ovalbumin Fusion Genes in MCF-7 Cells[a]

fusion gene	hormones	av % conversion
pMMTVCAT	I	0.30 ± 0.05
	I + Dex	51.88 ± 0.13
pOvCAT-1.3	I	0.59 ± 0.03
	I + Dex	0.41 ± 0.07
	I + E	0.64 ± 0.10
	I + Dex + E	0.80 ± 0.14
pOvCAT-.88	I	0.26 ± 0.03
	I + Dex + E	0.17 ± 0.04
pOvCAT-.1	I	8.98 ± 0.06
	I + Dex + E	5.68 ± 0.12
pOvCAT-.08	I	7.51 ± 0.04
	I + Dex + E	6.51 ± 0.14

[a] The indicated genes were transfected into MCF-7 cells, and the cells were cultured for 2 days in hormones as indicated. I = insulin at 50 ng/mL; E = estrogen and Dex = dexamethasone, both at 1×10^{-7} M. The data are the results of duplicate dishes ± the range of values.

hormonal conditions. Immediately after isolation, tubular gland cells were transfected with pOvCat-1.3, washed, pooled, and then aliquoted into culture dishes containing hormones as indicated in Figure 7. After 48 h, the cells were harvested; total nucleic acid for solution hybridization was prepared from some dishes, and cell extracts for CAT assays were prepared from replicate dishes. With only insulin in the culture medium, expression of both the exogenous and endogenous ovalbumin genes was very low. In contrast, when estrogen, corticosterone, and insulin were administered, expression of both genes was increased more than 100-fold. Estrogen and insulin alone stimulated little expression of either gene, while corticosterone or progesterone plus insulin induced expression to about 40% of that with both steroids. Physiological concentrations of insulin in addition to steroids are essential for maximal expression as depicted in the last two columns. These results indicate that the hormonal regulation of pOvCAT-1.3 in the tubular gland cells is comparable to that of the endogenous ovalbumin gene, suggesting that this construct encompasses the DNA regulatory elements that mediate the response to progesterone and corticosterone as well as to estrogen.

Expression of the Ovalbumin Promoter in Heterologous Cells. Although the ovalbumin gene is normally expressed only in tubular gland cells, it was of interest to determine whether the SRE and NRE regulatory elements are operative in other cell types. Some of the OvCAT fusion genes were introduced into MCF-7 cells, a human mammary cell line with estrogen and glucocorticoid receptors (Horwitz et al., 1975). For comparison, MMTVCAT, a gene that is known to be expressed in MCF-7 cells, was also transfected. The MCF-7 cells were cultured for several generations in medium containing serum that had been stripped of steroids. After transfection, steroids were added as indicated in Table I to the medium, and the cells were cultured for an additional 2 days. The level of expression of MMTVCAT was induced dramatically (173-fold) by dexamethasone. In contrast, expression of pOvCAT-1.3 was low when cultured with insulin and was not increased upon addition of dexamethasone, estrogen, or a combination of the two steroids.

Deletion analysis revealed that sequences between −1.3 and −.88 had no effect on the expression of the ovalbumin gene in MCF-7 cells. However, when the sequences upstream of −100 were removed (pOvCAT-.1 and pOvCAT-.08), expression increased substantially (33-fold increase compared to pOvCAT-.88). This expression was not inducible by steroids. These data suggest that the NRE represses expression of the ovalbumin promoter in heterologous cells as well as in oviduct cells but that an essential factor other than the steroid receptor

is missing from heterologous cells.

Discussion

The transfer of mutated genes into cells is widely employed to assess the functional relevance of specific DNA sequences and to define important regulatory elements. However, our initial attempts to apply this technique to the study of hormonal responses in primary chick oviduct cells failed because the transfection process terminated the response of both endogenous and exogenous genes to steroid hormones. In order to circumvent this problem, we have developed a method for transfecting primary oviduct cell cultures that does not disrupt expression of the endogenous ovalbumin gene. The key to retaining cellular responsiveness is to transfect the cells immediately after isolation and before attachment to culture dishes. Transfection even 1 or 2 days after the cells are cultured disrupts the accumulation of endogenous ovalbumin mRNA (Figure 1), and the expression of the OvCAT fusion genes is no longer steroid dependent. To further confirm that the OvCAT genes are regulated correctly, we compared their expression to that of the endogenous ovalbumin gene with various hormonal treatments (Figure 7). In all cases, induction of the transfected OvCAT genes in tubular gland cells accurately reflects the hormonal regulation of the endogenous ovalbumin gene.

Deletion analysis of OvCAT fusion genes demonstrated that sequences upstream of −880 are not essential to obtain maximal induction by steroids (Figure 5). However, when sequences between −880 and −520 were removed, expression was very low and could no longer be increased by steroids. Surprisingly, deletion of sequences between −350 and −100 completely restored high levels of expression that were steroid independent. Because the ovalbumin promoter could be repressed with ovalbumin sequences −584 to −248 (Figure 6B), the NRE probably resides between −350 and −248. Similarly, induction of the repressed promoter was restored with a fragment of DNA containing ovalbumin sequences −1154 to −585 (Figure 6B), indicating that the SRE resides between −880 and −585. These results suggest that expression of the ovalbumin gene is repressed by the NRE and that the steroid–receptor complex induces expression of the gene by relieving this repression, either directly or indirectly, through interactions involving sequences between −880 and −585.

The NRE may be operative in nonoviduct cells. When OvCAT fusion genes containing both the NRE and SRE (pOvCAT-1.3 or pOvCAT-.88) were transfected into MCF-7 cells, a human breast cell line with steroid receptors (Horwitz et al., 1975), no expression was observed (Table I). However, deletion mutants without the NRE (pOvCAT-.1 and pOvCAT-.08) were actively expressed but were not dependent upon steroids. This demonstrates that the NRE can prevent expression of the ovalbumin fusion gene in nonoviduct cells. In addition, because steroids cannot overcome this repression in MCF-7 cells, a tissue-specific factor other than the estrogen receptor must be present in oviduct tubular gland cells that is essential for steroid-dependent relief of repression.

Previous reports have yielded conflicting results on the nature and location of control elements in the ovalbumin promoter. In experiments undertaken in Chambon's laboratory (Chambon et al., 1984; Gaub et al., 1987), the ovalbumin promoter was fused to the SV40 T-antigen structural gene and this construct microinjected into primary cultures of chick oviduct cells. They reported high constitutive expression with genes containing the ovalbumin promoter and sequences to −132 or −295, in agreement with our results for constructs lacking the NRE. However, they reported some steroid responses to both estradiol and progesterone with a construct containing sequences to −420. In our experiments, constructs spanning this position (pOvCAT-.52 and pOvCAT-.35) showed low basal expression and no steroid response. Studies from O'Malley's laboratory (Dean et al., 1983, 1984) reported induction of an ovalbumin/globin fusion gene by both estrogen and progesterone in cultured oviduct cells and identified the steroid-responsive sequences as residing between −197 and −95. Their constructs also contained the SV40 enhancer which may have influenced the potential activities of the SRE and NRE we found in our experiments. In addition, all the previous studies mentioned were done on primary oviduct cultures after several days in culture, which may have altered their ability to respond to hormones. From our experience, we would not expect a response from either the endogenous ovalbumin gene or the transfected DNAs after this preincubation in culture. Thus, we suspect that the discrepancies in the location of the steroid response element are primarily due to differences in the steroid responsiveness of the primary oviduct cell cultures as prepared in the three laboratories, although differences in constructs and assay systems may also contribute.

Our data indicate that the dominant SRE is between −880 and −585, which coincides with a steroid-dependent DNase I hypersensitive site centered between −1.03 and −0.65 (Kaye et al., 1986). This hypersensitivity reflects a local reorganization of nucleosome structure and may be a direct result of protein binding to the SRE. In addition, the responsiveness of pOvCAT-.35 to steroids can be completely restored by adding a fragment of DNA containing sequences −1154 to −585 (Figure 6B), although this is orientation dependent. Thus, while the region of DNA between −880 and −585 can confer inducibility by steroids, it does not act like a typical enhancer; it may be that the spacing and orientation of the SRE relative to the NRE are important for relief of repression.

While negative regulation of gene expression is a common mechanism of gene control in procaryotes and in yeast [for reviews, see Brent (1985) and Reznikoff et al. (1985)] only relatively recently has this been documented in higher eucaryotes. Cis-acting negative regulatory sequences have now been defined for several genes. Some of these including sequences in the insulin 1 gene (Nir et al., 1986), the α-fetoprotein gene (Muglia & Rothman-Denes, 1986), the albumin gene (Petit et al., 1986), retinol binding protein genes (Colantuoni et al., 1987), and several viral enhancers (Gorman et al., 1985) prevent expression of the gene in cells that do not normally express the gene; these negative regulatory elements therefore operate in a tissue-specific or differentiation-dependent manner. Other cis-acting negative regulatory sequences such as those in the human β-interferon (Goodbourn et al., 1986) and P1-450 genes (Gonzalez & Nebert, 1986; Jones et al., 1985) may influence positive regulatory elements through protein interactions and thus play a direct role in gene regulation. The NRE in the ovalbumin gene appears to fall into the latter category. Ovalbumin mRNA levels and transcription rates are extremely low without steroids, indicating that there is virtually no basal transcription (Palmiter et al., 1981). This is consistent with the contention that the NRE represses transcription in the absence of steroids and that steroids act to restore transcriptional activity by relieving this repression. The mechanism of this negative regulatory effect is not known, but it may provide a means by which the ovalbumin gene is rapidly repressed when the inducing stimulus, the steroid, is withdrawn.

The discovery of a NRE in the ovalbumin gene may relate to earlier observations that fragments of DNA from MMTV

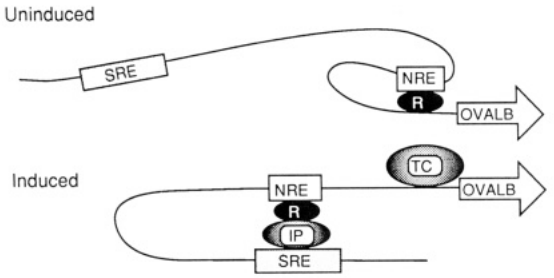

FIGURE 8: Proposed model for the regulation of the ovalbumin promoter. Two hypothetical states of the ovalbumin gene are shown, the basal and steroid-induced states. We propose that a repressor protein (R) binds to the NRE, thereby suppressing transcription by preventing the interaction of the transcription complex (TC) with the promoter. Upon treatment with steroids, a protein with a short half-life (IP) is induced that binds to the SRE. The interaction of the IP with the SRE initiates contact with R such that the TC can now initiate transcription of the ovalbumin gene.

containing glucocorticoid response elements may have an inhibitory effect when steroids are absent (Ostrowski et al., 1984; Overhauser & Fan, 1984). This may alter our perception of how steroids regulate gene expression. In addition to acting directly to increase the rate of transcription of target genes, steroids may also act indirectly to overcome the repressive effects of other regulatory proteins. Several additional lines of evidence suggest that the induction of the ovalbumin gene by steroids is a "secondary" event. These include the observations that the induction of transcription requires continuous protein synthesis (McKnight, 1978; McKnight et al., 1980) and is preceded by a significant lag (Palmiter, 1972; Palmiter et al., 1976). Analysis of the sequences in the SRE reveals no significant sequence homologies with the estrogen response element in the vitellogenin gene (Klein-Hitpass et al., 1986), with the glucocorticoid response element (identified in several genes) [for a review, see Yamamoto et al. (1985)], or with the progesterone response element (Cato et al., 1986; Strahle et al., 1987). Additionally, the SRE defined in these deletion analyses cannot confer steroid-dependent regulation on the heterologous TK promoter. Lastly, the SRE identified in our studies does not appear to act like a transcriptional enhancer as do the estrogen and glucocorticoid response elements.

While these functional studies have identified two regulatory elements, one positive and one negative, that modulate the ovalbumin promoter, neither of these elements alone confers their respective functions to a heterologous viral promoter. This suggests either that these are conditional regulatory elements and are therefore depending upon the specific promoter for activity or that they act as a single functional unit. Preliminary results indicate that when the SRE and NRE are both fused to the TK promoter that it now becomes regulated by steroids, supporting the idea that they must act in concert. However, it would not be surprising if these elements are most effective with their homologous promoter as the complex protein–protein interactions that occur at or near promoters in response to a particular inducer are promoter specific (Ptashne, 1986; Cordingly et al., 1987). Furthermore, the functional capacity of a particular regulatory element depends upon the promoter involved (DeFranco et al., 1985). Although in most cases regulatory elements can be successfully transferred to heterologous promoters, the level of activity observed is often significantly reduced (Ostrowski et al., 1984; Overhauser & Fan, 1984).

One simplistic model of how the SRE and NRE regulate the ovalbumin gene is depicted in Figure 8. We propose that in the absence of steroids, basal expression of the ovalbumin promoter is repressed through the binding of a protein (R) that is not tissue specific to sequences between −350 and −248 (the NRE). It is likely that this protein, or another protein interacting with it, makes direct contact with proximal transcription signals such as the "coup" box between −70 and −90 (Sagami et al., 1986), preventing the formation of atranscriptional complex (TC). Since induction of the gene by steroids requires concomitant protein synthesis, we suggest that a steroid-inducible protein (IP) with a short half-life acts at sequences between −880 and −585 (the SRE). The short-lived protein may be species or tissue specific as steroids are ineffective in activating the ovalbumin promoter in MCF-7 cells (Table I). We propose an interaction between the induced protein bound to the SRE and the protein bound to the NRE such that transcriptional inhibition of the ovalbumin promoter is relieved. Similar interactions between proteins at a distance on DNA have been suggested from work on procaryotes [for a review, see Ptashne (1984)]. A better understanding of the multihormonal regulation of the ovalbumin gene awaits additional characterization of the SRE and NRE and the proteins that interact with them.

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Expression of Human Angiogenin in Cultured Baby Hamster Kidney Cells†

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ABSTRACT: Baby hamster kidney cells were transformed with DNA sequences derived from the gene for human angiogenin. Expression was under the transcriptional control of the inducible mouse metallothionein 1 promoter. Recombinant angiogenin was purified and shown to be chemically, biologically, and enzymatically indistinguishable from the natural product. The large-scale production of recombinant angiogenin achieved should facilitate detailed studies into the structure–function relationships of this potent angiogenic molecule.

Angiogenin, a single-chain cationic polypeptide of M_r 14124, is a potent stimulator of blood vessel formation in both the chick chorioallantoic membrane and rabbit cornea (Fett et al., 1985). Originally isolated from medium conditioned by human colon adenocarcinoma cells (Fett et al., 1985), angiogenin has recently been detected in and isolated from normal human plasma (Shapiro et al., 1987). Thirty-five percent of its sequence is identical with that of human pancreatic ribonuclease (Strydom et al., 1985), and it, in fact, exhibits ribonucleolytic activity which is characteristic of and differs distinctly from that of pancreatic ribonuclease (Shapiro et al., 1986). The chemical structures of its gene and cDNA, isolated from normal liver libraries, have been determined (Kurachi et al., 1985).

Until recently there has not been enough purified angiogenin to carry out detailed investigations of its physiological mechanisms, interactions with substrates and/or target cells and tissues, and antigenic properties as well as potential clinical applications. In order to obtain sufficient material necessary for such studies, mammalian cell expression systems were investigated. We here report the successful engineering of such

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