MOLECULAR AND CELLULAR BIOLOGY, Mar. 1988, p. 1123–1131
0270-7306/88/031123-09$02.00/0

Vol. 8, No. 3

Two Functional Estrogen Response Elements Are Located Upstream of the Major Chicken Vitellogenin Gene

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Received 28 August 1987/Accepted 30 November 1987

We used a transient-expression assay to identify two estrogen response elements (EREs) associated with the major chicken vitellogenin gene (VTGII). Each element was characterized by its ability to confer estrogen responsiveness when cloned in either orientation next to a chimeric reporter gene consisting of the herpes simplex virus thymidine kinase promoter and the chloramphenicol acetyl transferase-coding region. Deletion analyses indicated that sequences necessary for the distal ERE resided within the region from −626 to −613 (nucleotide positions relative to the VTGII start site) whereas those necessary for the proximal ERE were within the region from −358 to −335. These distal and proximal elements contain, respectively, a perfect copy and an imperfect copy of the 13-base-pair sequence that is an essential feature of the EREs associated with two frog vitellogenin genes. These chicken VTGII EREs mapped near regions that were restructured at the chromatin level when the endogenous VTGII gene was expressed in the liver in response to estradiol. These data suggest a model for the tissue-specific expression of this estrogen-responsive gene.

The ability of the White Leghorn chicken to lay a 50-g egg daily represents a rather impressive biological feat. Making the large amount of protein that goes into the egg involves two discrete tasks. The egg white proteins (i.e., ovalbumin, conalbumin, lysozyme, and ovomucoid) are synthesized in the oviduct (for reviews, see references 6, 22, 33, and 34). In contrast, the production of the egg yolk proteins, which include two apolipoproteins (apoB and apoVLDLII) and the three vitellogenin proteins (VTGI, VTGII, and VTGIII), is accomplished in the liver (for reviews, see references 10, 39, 42, and 45). Despite the fact that the oviduct and liver are each very large tissues, the magnitude of the effort required to produce an egg at such frequency is reflected by the fact that in the laying hen the respective egg protein RNAs are among the most abundant messages in either tissue. Elevated levels of estrogen coordinate the synthesis of these two sets of proteins and confine their expression to periods of egg laying. In the case of yolk protein genes, this expression occurs only in response to estradiol, although in the differentiated oviduct, a broader spectrum of steroids can modulate the expression of egg white protein genes. A major goal of many laboratories over the last several decades has been to determine how these steroid- and tissue-specific programs of gene expression are encoded at the molecular level.

Our understanding of the regulation of yolk protein genes has been greatly facilitated by the fact that, although they are normally expressed exclusively in hens during periods of egg laying, a single injection of estradiol elicits the expression of this battery of genes in birds of either sex (1, 11, 23). This expression can be effected as early as day 9 of embryonic development and correlates roughly with the time when the expression of estrogen receptors is first demonstrable in the avian liver (14).

Although coordinately regulated by estradiol at a gross level, the individual yolk protein genes differ greatly with respect to their specific response characteristics. First, the genes display distinct developmental profiles in response to estradiol. For example, the apoB gene can be maximally expressed as early as day 9 of embryonic development (16), whereas the VTGI gene cannot be maximally expressed until birds are 6 weeks of age (15). Since the level of estrogen receptors increases over this period (14, 15), the different yolk protein genes may require distinct threshold levels of receptors for their maximal expression. Second, the maximal expression of the genes differs by as much as an order of magnitude: 30,000 copies of VTGII mRNA are present in the liver of a laying hen compared with only 3,000 copies of VTGIII mRNA (16, 21, 23, 47). Third, the time that elapses between the addition of estradiol and the rapid accumulation of RNA is distinct for each of the yolk protein genes (8, 11, 16, 23). This is true after both primary and secondary injections of estradiol, although in the latter case, the four genes that are induced de novo by estradiol (i.e., apoVLDII and the three vitellogenin genes) are each expressed more rapidly (8, 11, 16, 23). This long-term (4) reprogramming event, which is effected in response to a single injection of estradiol, is known as memory (1).

To understand the estrogen-dependent tissue-specific regulation of the chicken egg white and egg yolk protein genes, it is essential to identify and characterize the DNA control elements associated with the respective genes. In the last several years, considerable progress has been made toward this goal (3, 6, 9, 12, 17, 24, 28, 36). In the present paper, we report detailed functional analysis of estrogen response elements (EREs) associated with a chicken yolk protein gene. We examined 1.1 kilobases (kb) of 5′-flanking DNA (3, 44) from the major vitellogenin gene (VTGII) and identified two functional EREs which share homology with the EREs described for the frog vitellogenin genes (25, 38). For the chicken VTGII gene, these EREs map to regions that we (3–5) and others (31, 37) previously identified as being restructured in vivo at the chromatin level in response to estradiol. On the basis of these data, we propose a model to account for the tissue-specific expression of this estrogen-responsive gene.

MATERIALS AND METHODS

Plasmid constructions. The parent chloramphenicol acetyltransferase (CAT) plasmid used for all the transient-expres-

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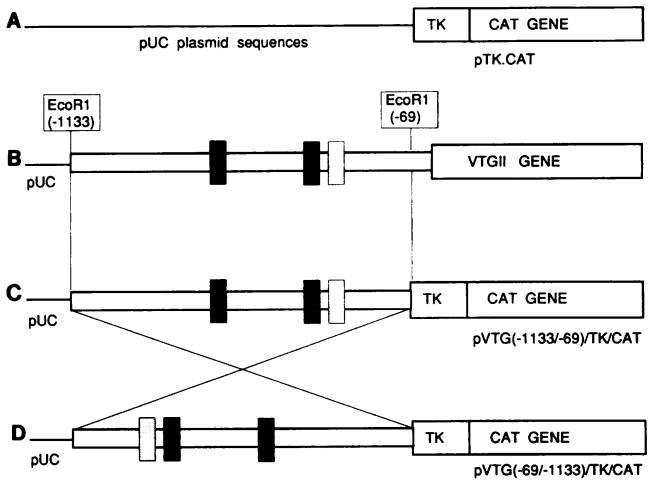

FIG. 1. Construction of the parental pVTG/TK/CAT plasmids. (A) pTK.CAT plasmid; (B) 5'-flanking region of the VTGII gene. (Only the TK promoter region and the 5'-flanking region of the VTGII gene are drawn to scale.) The *Eco*RI fragment from −1133 to −69 was cloned (by using *Hin*dIII linkers) in each orientation upstream of the TK promoter in pTK.CAT to yield pVTG (−1133/−69)/TK/CAT (C) and pVTG(−69/−1133)/TK/CAT (D). The *Hin*dIII site proximal to the TK promoter was then eliminated to leave a unique promoter-distal *Hin*dIII site for each construct. This *Hin*dIII site and a unique promoter-proximal *Bam*HI site located in the polylinker between the VTGII insert and the TK promoter were then used as entry sites for creating the series of BAL 31 deletion constructs presented in Fig. 2 to 5. The sequences previously identified as homologous to the consensus ERE sequence (46) are indicated by vertical boxes within the VTGII sequence. The two elements shown by the present study to be functional (see Fig. 2 to 5) are indicated by dark grey shading, whereas the other copy is indicated by light grey shading.

sion assays is pTK.CAT (a generous gift from G. Schutz and R. Miksicek; Fig. 1). This plasmid is a chimeric construct with the herpes simplex virus thymidine kinase (TK) promoter and start site (−105 to +51) fused to the CAT-coding region, which in turn is fused to the splice and polyadenylation signals from simian virus 40. The CAT-splice-polyadenylation cassette was taken from pSV2.CAT (18), whereas the TK promoter was derived from the 115-to-105 linker scanning mutant of McKnight and Kingsbury (30). Immediately upstream of the TK promoter are three unique cloning sites (*Hin*dIII, *Sal*I, and *Bam*HI) from pUC8. The two parent pVTG/TK/CAT plasmids used in this study were constructed from pTK.CAT as follows. The 1.1-kb *Eco*RI fragment located immediately upstream of the VTGII promoter was isolated from plasmid pVTG104 (3). This fragment extends from the *Eco*RI site at −69 to an *Eco*RI linker at −1133; the latter is the left end of the lambda clone insert from which the fragment was originally subcloned (5). (Nucleotide positions are numbered relative to the VTGII start site [44].) The *Eco*RI ends were filled by using the Klenow fragment of *Pol*I; then, *Hin*dIII linkers were added, using T4 ligase, and the fragment was cloned in both orientations into the *Hin*dIII site of pTK.CAT. (Unless otherwise referenced, standard protocols were followed for all the DNA manipulations described in this report [29].) The *Hin*dIII site proximal to the TK promoter was then mutated for each of the two constructs by a partial *Hin*dIII digest, followed by filling in with Klenow fragment and religation. These plasmids were designated pVTG(−1133/−69)/TK/CAT and pVTG (−69/−1133)/TK/CAT.

We created a series of 5' deletions for each of the two pVTG/TK/CAT clones described above by digesting the clones with *Hin*dIII and incubating them with BAL 31 for various periods of time. Samples were pooled and treated with Klenow fragment to make the ends blunt, and *Hin*dIII linkers were added. The inserts were then released by digestion with *Hin*dIII and *Bam*HI, and the collection of fragments was isolated from an agarose gel and directionally cloned into pTK.CAT. The BAL 31 endpoints were determined for the supercoiled plasmids (7) by using the method of dideoxy sequencing and an M13 universal primer directed against the pUC8 sequences located immediately upstream of the *Hin*dIII site on pTK.CAT. Similarly, 3' deletions were engineered from the unique *Bam*HI site for each of the two parent pVTG/TK/CAT constructs. These junctions were sequenced by using a primer (5' ACTGCATCTGCGT GTTCG3') directed against the TK promoter (−58 to −75).

As exceptions, the −725 and −613 endpoints were obtained by using restriction enzymes (*Pst*I and *Msp*I, respectively) rather than BAL 31 nuclease. The −1133/−725 and −1133/−613 fragments were cloned into the *Hin*dIII site of pTK.CAT by using linkers and thus differed from the BAL 31 deletions described above in that they retained the short pUC8 *Hin*dIII-*Bam*HI polylinker that was located immediately upstream of the TK promoter.

Transient-expression assays. The plasmids used for transfection were prepared by the alkaline lysis method followed by purification through CsCl gradients. Each plasmid isolated in this way was predominantly supercoiled, as judged by gel electrophoresis. DNA concentrations were determined by fluorescence spectroscopy (27). HepG2 cells (26) were grown in Dulbecco modified Eagle medium-F12 (1:1, vol/vol) medium containing 10% steroid-stripped fetal calf serum (40), 10 μg of insulin per ml, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 100 μg of kanamycin per ml. The calcium phosphate method (19) was used to cotransfect 10 μg each of the pKCR2-ER (20) and CAT plasmids into HepG2 cells grown on 60-mm-diameter dishes. (A description of pKCR2-ER is provided in Results.) Transfected cells were cultured for 40 h in the presence or absence of 10^{-6} M moxestrol (replenished every 12 h). This estrogen analog gave results comparable with those obtained with estradiol (data not shown) and was used in place of estradiol because it is metabolically more stable (35). In the present report, we will henceforth refer to the expression effected by moxestrol as estrogen-dependent expression. The cells were harvested, and CAT assays were performed (18) with a fixed amount of protein for each series. Protein concentrations were determined by the method of Bradford (2); the amount of protein assayed for CAT activity was either 50 or 150 μg depending on the experiment. After chromatography, the silica plates were sprayed with En^{3}Hance (Du Pont Co.) and exposed to X-Omat film (usually overnight) at −70°C with Lightning-Plus intensifying screens. The extent of chloramphenicol acetylation was quantitated by cutting the appropriate regions from the plates and counting them in scintillation fluid. These values were normalized relative to the CAT activity present in the pTK.CAT-transfected cells.

RESULTS

We cloned the −1133/−69 fragment from the 5'-flanking region of the VTGII gene (3, 44) in both orientations immediately upstream of the TK promoter in pTK.CAT (Fig. 1). These two plasmids were transfected into HepG2 cells (26)

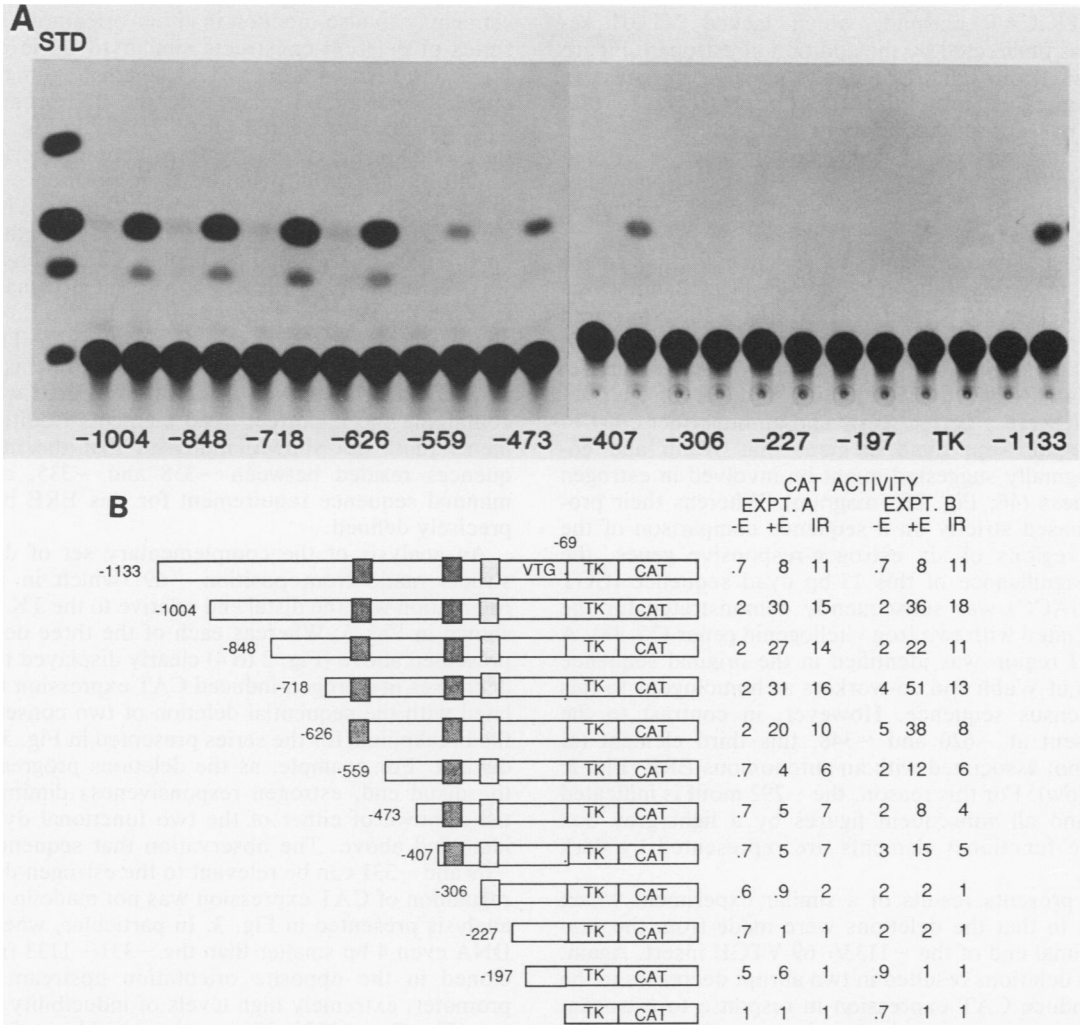

FIG. 2. Estrogen-dependent CAT expression from a series of constructs containing various portions of VTGII flanking DNA cloned upstream of the TK promoter: 5′ deletion series. pVTG/TK/CAT constructs were cotransfected into HepG2 cells with an expression vector for the human estrogen receptor. The cells were cultured in the presence or absence of estrogen, and then extracts were prepared and assayed for CAT activity. (A) Autoradiogram of CAT activity. For each plasmid, the left or right lanes indicate the CAT activity observed when the transfected cells were grown in the absence or presence of estrogen, respectively. The pair of lanes labeled TK represents cells transfected with the pTK.CAT plasmid, which lacks VTGII sequences. The spots were cut out of the lanes and counted. STD, CAT standard. (B) Quantitative data from this experiment (EXPT. A), as well as data obtained from an independent experiment using the same constructs (EXPT. B), and a schematic representation of the VTGII sequences present in each of the deletion constructs (the CAT gene is not drawn to scale). Sequences related to the ERE consensus motif (46) are indicated by shaded boxes in the VTGII flanking region. Dark grey shading indicates the two motifs associated with functional EREs, whereas light grey shading indicates the third sequence which was not. CAT activity was normalized for each experiment relative to the activity obtained in the cells transfected with pTK.CAT. −E, Without estrogen; +E, with estrogen; IR, induction ratio for each construct obtained by calculating the ratio of the CAT activity in the presence of estrogen to that obtained in the absence of estrogen.

and assayed for their ability to transiently express CAT in the presence or absence of estrogen (see Materials and Methods for details). An expression vector (pKCR2-ER) coding for the human estrogen receptor (20) was cotransfected with these pVTG/TK/CAT constructs to supplement the low endogenous receptor levels present in HepG2 cells (41). Since this VTGII fragment conferred estrogen responsiveness to the otherwise insensitive pTK.CAT plasmid (see below), deletion constructs were made to map the EREs within this flanking region of the VTGII gene.

An autoradiogram showing the transient CAT expression obtained from a series of 5′ deletion constructs in which VTGII flanking sequences were cloned upstream of the TK promoter in the same orientation as they normally reside upstream of the VTGII start site is shown in Fig. 2A. The CAT standard at the extreme left shows the positions of the chloramphenicol substrate (the bottom spot), the two monoacetylated derivatives (the two middle spots), and the diacetylated derivative (the top spot). The CAT enzyme levels in extracts from the transfected cells were such that, under our assay conditions, the chloramphenicol was converted to the two monoacetylated forms only. The construct containing the entire −1133/−69 VTGII fragment [henceforth denoted pVTG(−1133/−69)/TK/CAT] is shown at the extreme right. In this experiment, the amount of CAT enzyme expressed from this plasmid increased approximately 10-fold by culturing the transfected cells in the presence of estrogen. In contrast, expression from the

parental pTK.CAT plasmid, which lacked VTGII sequences, was unaffected by the addition of estrogen. Figure 2 also shows (from left to right) the effect of progressive deletions from the 5′ end of the VTGII insert. It is clear that there were two abrupt decreases in estrogen-induced CAT expression; the removal of sequences between −626 and −559 reduced induction, whereas the subsequent removal of sequences between −407 and −306 abolished induction completely.

These data and data from an independent experiment were quantitated, and the results are shown in Fig. 2B. First, the deletion of sequences between −1133 and −1004 appeared to relieve a restraint on the magnitude of induction. Second, the critical breakpoints with respect to estrogen responsiveness correlated with the sequential removal of two sequences that were a perfect copy and an imperfect copy of the 13-base-pair (bp) dyad element that Wahli and co-workers originally suggested might be involved in estrogen responsiveness (46; Fig. 2B, diagram). Whereas their proposal was based strictly on a sequence comparison of the 5′-flanking regions of six estrogen-responsive genes, the functional significance of this 13-bp dyad sequence (GGT CANNNTGACC) was subsequently demonstrated for the EREs associated with two frog vitellogenin genes (25, 38). A third VTGII region was identified in the original sequence comparison of Wahli and co-workers as homologous to this 13-bp consensus sequence. However, in contrast to the copies present at −620 and −348, this third element (at −292) was not associated with an autonomous ERE (Fig. 2; see also below). For this reason, the −292 motif is indicated in Fig. 2 and all subsequent figures by a light grey box whereas the functional elements are represented by dark grey boxes.

Figure 3 presents results of a similar experiment which differs only in that the deletions were made from the promoter-proximal end of the −1133/−69 VTGII insert. Again, progressive deletions resulted in two abrupt decreases in the ability to induce CAT expression in response to estrogen. The first decrement occurred when the region between −335 and −434 was deleted and resulted in a dramatic loss of estrogen responsiveness. As additional sequences were removed (which also brought the upstream consensus element closer to the TK promoter), the response to estrogen increased somewhat, but the removal of sequences between −613 and −725 resulted in the complete loss of estrogen-dependent enhancement of CAT expression. Again, these breakpoints coincided with the sequential removal of the two GGTCANNNTGACC-related elements implicated in the previous experiment. With the results presented in Fig. 2, these data set limits on the VTGII sequences required to confer estrogen responsiveness. In the case of the upstream element, the region bracketed by these deletions was only 14 bp in size, of which 13 bp were the dyad element itself. The sequences necessary for the promoter-proximal response element are more precisely delineated below. It is evident that the expression of CAT was depressed for some of the pVTG/TK/CAT constructs assayed in the absence of estrogen, relative to what was observed for pTK.CAT (Fig. 3). Similar results have been obtained with other transient-expression assays (for example, see Fig. 4 and Table 1 below). We do not understand the significance of this variable repression.

Since the data in Fig. 3 indicate that these EREs can function from different positions relative to the TK promoter, they display one of the hallmarks of enhancer elements (but see also below). To determine whether these elements can also function in either orientation, we tested a series of deletion constructs similar to those presented in Fig. 2 and 3 but with the VTGII flanking region cloned in the opposite orientation relative to the TK promoter. These deletions are represented schematically in Fig. 4 and 5 with the results of the respective transfection experiments.

With the series of deletions from position −1133 (which for this set of constructs was proximal to the TK promoter), once again a two-step decrease in estrogen inducibility was apparent, with each step occurring as a consensus sequence was deleted (Fig. 4). In addition, it seemed that the magnitude of the response increased as the pair of functional consensus elements was moved closer to the TK promoter. The locations of the deletion endpoints in this experiment better defined the region of DNA associated with the ERE containing the imperfect dyad element. Additional experiments (data not shown) indicated that the necessary sequences resided between −358 and −335, although the minimal sequence requirement for this ERE has not been precisely defined.

An analysis of the complementary set of deletion constructs made from position −69 (which in this reverse orientation was the distal end relative to the TK promoter) is shown in Fig. 5. Whereas each of the three deletion series presented above (Fig. 2 to 4) clearly displayed two stepwise decreases in estrogen-induced CAT expression which correlated with the sequential deletion of two consensus motifs, the breakpoints for the series presented in Fig. 5 were not as distinct. For example, as the deletions progressed in from the distal end, estrogen responsiveness diminished before the removal of either of the two functional dyad elements identified above. The observation that sequences between −69 and −331 can be relevant to the estrogen-dependent up regulation of CAT expression was not made in the previous analysis presented in Fig. 3. In particular, when a piece of DNA even 4 bp smaller than the −331/−1133 fragment was cloned in the opposite orientation upstream of the TK promoter, extremely high levels of inducibility were apparent (Fig. 3, −1133/−335 construct). Thus, the sequences between −69 and −331 are not essential for estrogen responsiveness but appear to be relevant under certain circumstances. The second conclusion from Fig. 5 is that the two constructs that retained only the −620 dyad element examined in this series were marginal in their ability to respond positively to the addition of estrogen. A comparable phenotype was apparent for two similar constructs examined in Fig. 3. On the basis of these two sets of data, it seems that this isolated ERE does not function efficiently if located at a distance from the TK promoter.

To explore further how distance might affect the ability of the EREs to confer estrogen-dependent expression, we also made a set of constructs in which the EREs were located at greater distances from the TK promoter. This was accomplished by cloning 0.7- and 1.5-kb fragments of random sequence DNA (in this case, cDNA fragments) into the *Bam*HI site located immediately upstream of the TK promoter in pVTG(−1133/−69)/TK/CAT, pVTG(−1133/−335)/TK/CAT, and pVTG(−1133/−613)/TK/CAT, as well as in pTK.CAT. All but one of these pVTG/TK/CAT plasmids retained the capacity to enhance CAT expression in response to estrogen (Table 1). The efficiency with which this was accomplished, however, differed for the three sets of constructs. For example, the estrogen response function encoded within the −1133/−69 fragment was quite insensitive to being displaced as much as 1.5 kb away from the TK promoter. In contrast, the −1133/−335 fragment (which also

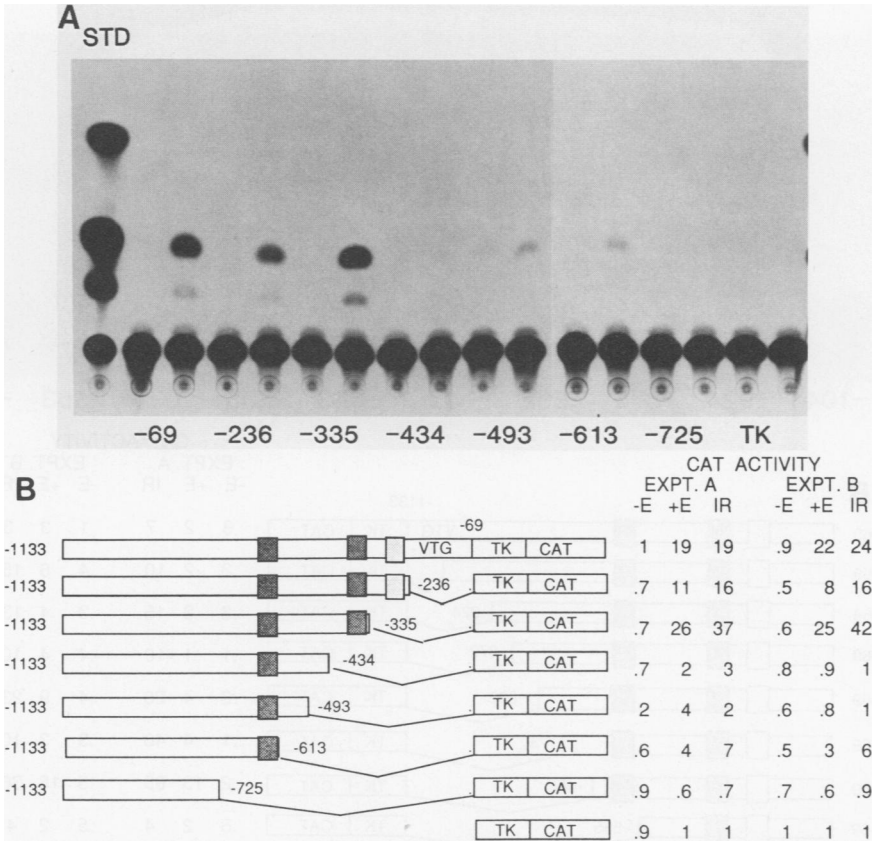

FIG. 3. Estrogen-dependent CAT expression from a series of constructs containing various portions of VTGII flanking DNA cloned upstream of the TK promoter: 3′ deletion series. (A and B) As described in the legend to Fig. 2, except that the deletions removed various portions of VTGII flanking DNA from the −69 end, which was proximal to the TK promoter in this set of constructs.

contained both EREs) functioned more efficiently than the −1133/−69 fragment when placed close to the TK promoter (Fig. 3) but less efficiently when displaced from the TK promoter by either 0.7 or 1.5 kb. Similarly, the −1133/−613 fragment, which contained only the distal ERE dyad, functioned much less efficiently when moved away from the TK promoter, in agreement with the results presented in Fig. 5.

Thus far, we have been unable to detect CAT RNA by using either primer extension (3) or RNase protection (49) protocols (data not shown). Nonetheless, there are two reasons why we think it likely that the VTGII EREs function in the present set of experiments by enhancing transcription from the heterologous TK promoter. First, CAT activity was abolished when the TK promoter was deleted from the parental pVTG/TK/CAT construct (data not shown). Second, Wahli and co-workers demonstrated that the functional ERE from the B1 vitellogenin gene of *Xenopus laevis* confers estrogen-dependent expression from the TK promoter when cloned into a similar pTK.CAT vector and assayed in MCF7 cells (38).

DISCUSSION

Our analysis of the 5′-flanking region of the major chicken vitellogenin gene (VTGII) revealed the first example of multiple functional EREs associated with a single gene. In transient-expression assays in which these VTGII EREs were analyzed in the context of a heterologous promoter, the two EREs functioned independently and in an orientation-independent fashion. In addition, when the two EREs were present on the same construct, the response was greater than what was observed with either element alone. Depending on the particular construct, the effect of the second ERE was very dramatic, indeed (Fig. 2 and 3).

In agreement with recent studies on the EREs associated with the frog A2 and B1 vitellogenin genes (25, 38), our data implicated GGTCANNNTGACC-related sequences as essential features of each of the two EREs associated with the chicken VTGII gene. The VTGII distal response element at −620 (ERE_D) contained a perfect copy of this dyad element (GGTCAGCGTGACC). A perfect, albeit distinct, copy of the dyad (GGTCACAGTGACC) is also an essential feature of the functional ERE located upstream of the frog A2 gene. In contrast, the chicken VTGII proximal response element at −348 (ERE_P) contains an imperfect match (GGTCAACA TAACC). The frog B1 functional element at −332 also contains an imperfect copy of the consensus sequence (AGTCACTGTGACC), but it is not clear whether this element is functional when analyzed out of the context of the other imperfect consensus sequence that resides near it (38). We have also made a construct related to pVTG(−1133/−613)/TK/CAT in which position 12 of the ERE_D dyad was changed (GGTCAGCGTGAAC), and we found that this solo consensus element remained functional as an ERE (data not

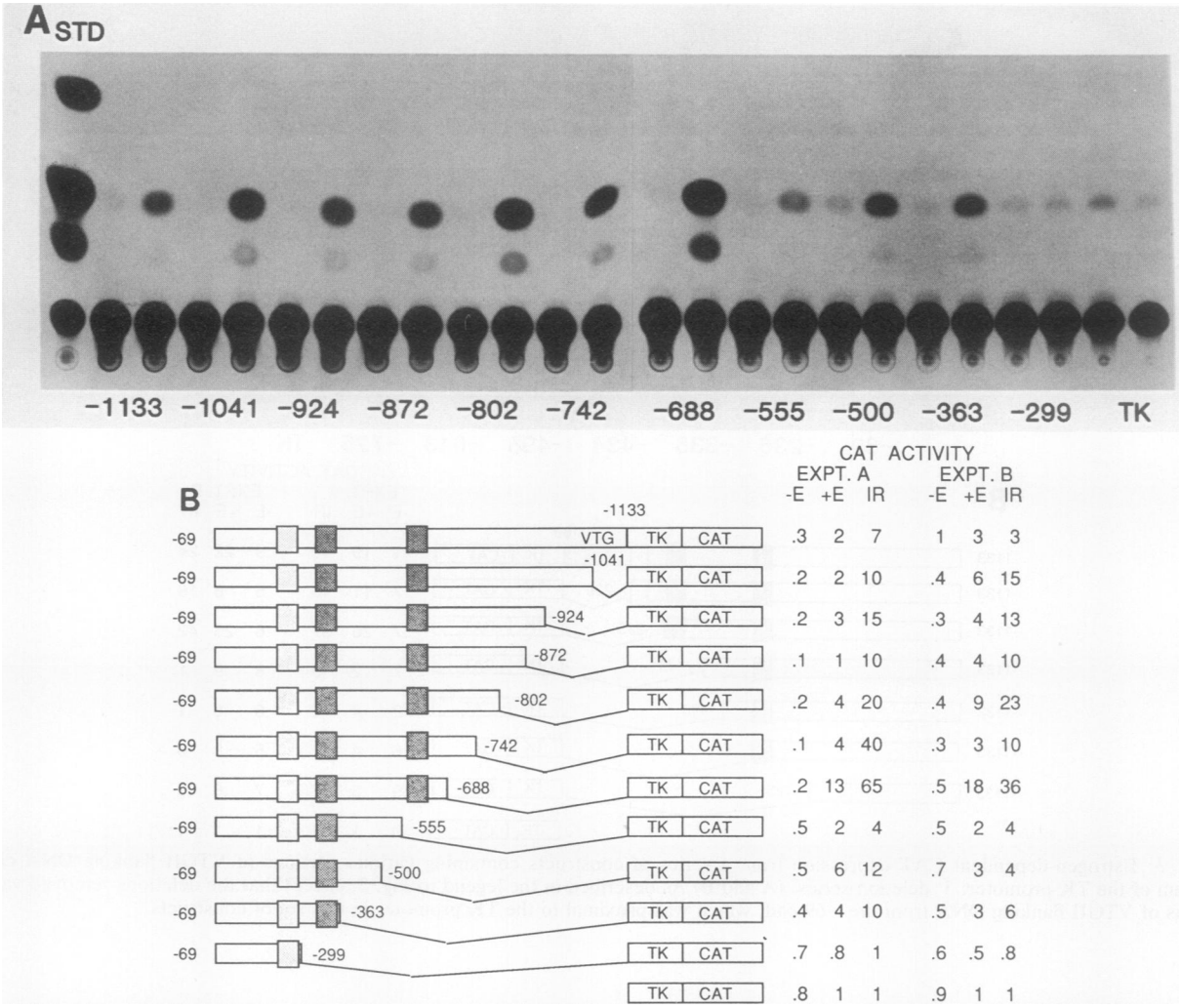

FIG. 4. Estrogen-dependent CAT expression from a series of constructs containing various portions of VTGII flanking DNA cloned in reverse orientation upstream of the TK promoter: 3′ deletion series. (A and B) As described in the legend to Fig. 2, except that the VTGII fragment from −1133 to −69 was cloned in the reverse orientation upstream of the TK promoter. Thus, deletions initiated at −1133 in the VTGII sequence occurred proximal to the TK promoter in this set of constructs.

shown). Thus, there appears to be some degree of tolerance for single-base changes within the consensus dyad.

On the other hand, not all of the sequences that have been identified by sequence analysis as homologous to the consensus dyad are functional as EREs, at least as determined by transient-expression assays. For example, Wahli and co-workers (46) noticed the presence of a third motif homologous to the consensus dyad upstream of the chicken VTGII gene, and yet our results provide no evidence to suggest that this element (at −292) is part of an autonomous ERE (Fig. 2 and 4). A similar situation has been reported for the frog B1 gene in which at least two of the three elements identified by sequence analysis are not by themselves functional as EREs in a transient-expression assay (38). Curiously, however, all three of these frog B1 sequence motifs can form stable protein-DNA complexes when incubated with nuclear extracts from estradiol-stimulated livers and analyzed by electron microscopy (43). These seemingly disparate results underscore the possibility that for the VTGII gene the third GGTCANNNTGACC-related motif (at −292), or even a fourth GGTCANNNTGACC-related motif (at −769; see below), could be important despite its apparent failure to function autonomously in a transient-expression assay. For example, it may be significant that the −292 motif resides within the region (from −335 to −69) that appeared to facilitate the ability of the two functional EREs to enhance CAT expression under certain circumstances (Fig. 5 and Table 1). Further experiments will be required to address this possibility.

It has been reported previously that a DNase I footprint can be detected over the region from −621 to −598 when purified VTGII flanking DNA is incubated with a crude preparation of estrogen receptors (24). This footprint spans a region including the right half of the ERE_D dyad plus an additional 16 bp of flanking DNA. We were surprised, therefore, to find that a deletion which removed two-thirds of the footprinted region did not abolish the activity of the ERE_D [Fig. 3, pVTG(−1133/−613)/TK/CAT]. The DNA sequence located immediately downstream of the dyad in this construct (see Materials and Methods) did not resemble the VTGII sequences that were footprinted in vitro. Furthermore, two additional constructs in which this region was

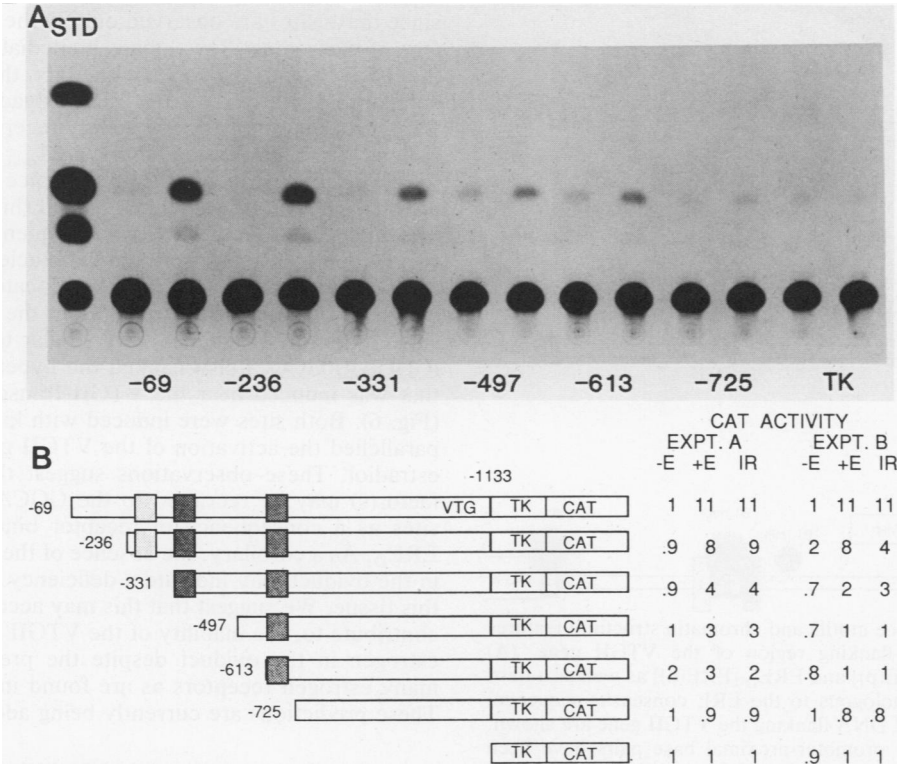

FIG. 5. Estrogen-dependent CAT expression from a series of constructs containing various portions of VTGII flanking DNA cloned in reverse orientation upstream of the TK promoter: 5′ deletion series. (A and B) As described in the legend to Fig. 2, except that the VTGII fragment from −1133 to −69 was cloned in the reverse orientation upstream of the TK promoter. Thus, deletions initiated at −69 in the VTGII sequence occurred distal to the TK promoter in this set of constructs.

replaced with other apparently unrelated sequences yielded levels of induction indistinguishable from those observed with the pVTG(−1133/−613)/TK/CAT construct (data not shown). Thus, our transient-expression assays failed to demonstrate that this portion of the footprint is of functional importance.

It is interesting to evaluate the locations of the functional ERE_P and ERE_D relative to regions that were restructured at the chromatin level when the endogenous VTGII gene was activated in response to estradiol (3–5; Fig. 6). First, it is striking that the single *Msp*I site that became demethylated within the VTGII gene domain in the liver in response to a single injection of estradiol (5, 31, 37, 48) overlapped the functional ERE_D dyad. The cytosine residue that was demethylated within this restriction site constitutes the last nucleotide of the consensus dyad. Indeed, a second cytosine

TABLE 1. CAT expression from constructs with or without 0.7- or 1.5-kb fragments of random DNA inserted immediately upstream of the TK promoter

Construct	CAT activity[a]					
	Expt A			Expt B		
	−E	+E	IR	−E	+E	IR
pVTG(−1133/−69)/TK/CAT	0.4	6	15	0.5	6	12
pVTG(−1133/−69)/0.7-kb insert/TK/CAT	0.3	4	13			
pVTG(−1133/−69)/1.5-kb insert/TK/CAT				0.7	5	7
pVTG(−1133/−335)/TK/CAT	0.2	9	45	0.2	9	45
pVTG(−1133/−335)/0.7-kb insert/TK/CAT	0.1	1	10			
pVTG(−1133/−335)/1.5-kb insert/TK/CAT				0.6	2	3
pVTG(−1133/−613)/TK/CAT	0.3	4	13	0.4	4	10
pVTG(−1133/−613)/0.7-kb insert/TK/CAT	0.1	0.6	6			
pVTG(−1133/−613)/1.5-kb insert/TK/CAT				0.7	1	1
pUC8/TK/CAT[b]	1	1	1	1	1	1
pUC8/0.7-kb insert/TK/CAT	2	2	1			
pUC8/1.5-kb insert/TK/CAT				1	0.8	0.8

[a] −E, Without estrogen; +E, with estrogen; IR, induction ratio as defined in the legend to Fig. 2.
[b] Parent plasmid pTK.CAT.

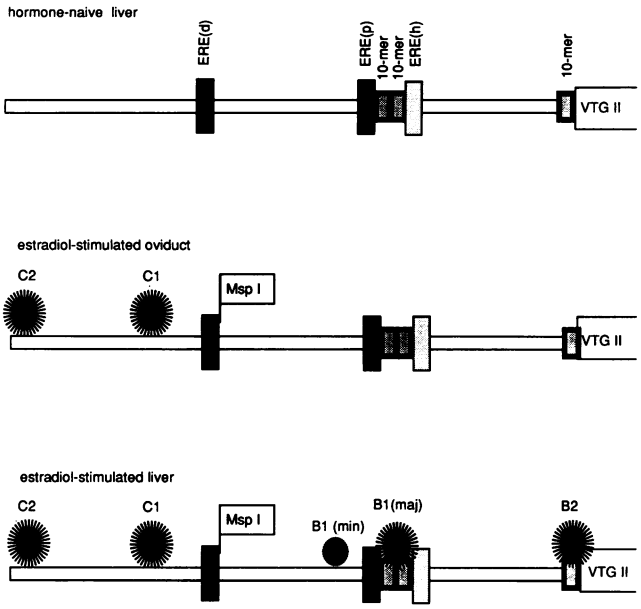

FIG. 6. DNA sequence motifs and chromatin structural features associated with the 5'-flanking region of the VTGII gene. (A) Locations of ERE_P [ERE(p)] and ERE_D [ERE(d)] as well as a third sequence [ERE(h)] homologous to the ERE consensus sequence. Approximately 1.1 kb of DNA flanking the VTGII gene are shown, of which all but the 69 promoter-proximal base pairs have been analyzed in the present study. The positions of the GGCARRAC CA-related 10-mer motifs are also indicated. Superimposed over these DNA sequence motifs are the chromatin structural features that characterize this gene region in the estradiol-stimulated oviduct (B) and estradiol-stimulated liver (C). These features were not apparent in the hormone-naive liver. The flag (Msp I) indicates that the *Msp*I restriction site, which overlapped ERE_D, was demethylated in response to estradiol. Star bursts indicate the positions of nuclease-hypersensitive sites. See text for details.

residue within the ERE_D dyad is also demethylated in response to estradiol (35). The only other tissue in which the demethylated form of the ERE_D dyad is observed is the oviduct of estrogen-stimulated birds. We interpret this to mean that the ERE_D very likely interacts with receptors in both oviduct and liver, even though the VTGII gene is expressed only in the liver. Additional indirect evidence consistent with the idea that receptors may interact with the VTGII locus in the oviduct is provided by the observation that two of the hypersensitive sites (C1 and C2) that were induced in the liver in response to estradiol are also observed in the stimulated oviduct (3). (Curiously, a sequence related to the ERE consensus sequence [i.e., GTTCTAGCTGACC] is present at the C1 hypersensitive site, although our transient-expression analyses failed to indicate that this sequence [at −769] was functional.) On the basis of transient-expression experiments that have been reported thus far, it does not appear that EREs per se are restricted in a tissue-specific fashion (13, 25, 38; unpublished results). Thus, we suggest that the failure of the resident VTGII gene to be expressed in the oviduct may reflect an inability of the VTGII promoter (or other regulatory element or both) to respond to these activated EREs in this cell type.

How might this differential activation of the VTGII promoter be effected at the molecular level? A clue may be provided by the three additional nuclease-hypersensitive sites ($B1_{maj}$, $B1_{min}$, and B2) that are induced in the 5'-flanking region of the VTGII gene in response to estradiol, since these sites are observed only in the liver (3, 5; Fig. 6). One of these sites ($B1_{maj}$) maps immediately downstream of the ERE_P dyad. However, the fact that there is not a comparable site next to the ERE_D dyad suggests that the $B1_{maj}$ site may not directly reflect receptor binding in this region. We suggest instead that the $B1_{maj}$ site may be related to two direct repeats of a 10-bp sequence (GGCARRACCA, in which R is a purine) which flanked this site (Fig. 6). The first copy of this GGCARRACCA sequence was located just two nucleotides away from the last nucleotide of the ERE_P dyad, whereas the second copy was located exactly one turn of the DNA helix downstream from the first. Moreover, a single copy of a sequence very similar to GGCARRACCA (GGCARRGCCC) also flanked the hypersensitive site (B2) that was induced near the VTGII transcriptional start site (Fig. 6). Both sites were induced with kinetics that roughly parallelled the activation of the VTGII gene in response to estradiol. These observations suggest that a transcription factor(s) may be recruited to the GGCARRACCA-binding sites as a consequence of receptor binding to ERE_P and ERE_D. As a corollary, the absence of the $B1_{maj}$ and B2 sites in the oviduct may indicate a deficiency of such a factor in this tissue. We suggest that this may account for, or at least contribute to, the inability of the VTGII gene to respond to estrogen in the oviduct despite the presence of twice as many estrogen receptors as are found in the liver (15, 32). These predictions are currently being addressed.

ACKNOWLEDGMENTS

We thank G. Schutz and R. Miksicek for the pTK.CAT plasmid, Pierre Chambon for the pKCR2-ER plasmid, Sally Camper for the HepG2 cells, Rein Strijker for suggesting the use of moxestrol in place of estradiol, and Secretarial Services for typing the manuscript. We also thank numerous colleagues for critically reading the manuscript.

This work was supported by Public Health Service grants CA06927, GM0335 (to J.B.), and postdoctoral training grant CA-09035 (to T.F.) from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

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