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Regulation of the Chicken Ovalbumin Gene by Estrogen and Corticosterone Requires a Novel DNA Element That Binds a Labile Protein, Chirp-I

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Because induction of the chicken ovalbumin (Ov) gene by steroid hormones requires concomitant protein synthesis, efforts have focused on defining the binding site in the Ov gene for a labile transcription factor. Previous gel mobility shift studies identified one such site in the steroid-dependent regulatory element (SDRE) between −900 and −853. To ascertain whether estrogen and glucocorticoid affect the binding of this labile protein, genomic footprinting of the Ov gene was done by treating primary oviduct cell cultures with dimethyl sulfate. Several alterations that include steroid-dependent protection of guanine residues −889 and −885 and hypersensitivity of adenine residues −892 and −865 were observed. Of particular importance, the in vivo footprinting data are corroborated by two functional studies, one with linker-scanning mutations and the other with point mutations. Ten-base-pair linker-scanning mutations between −900 and −878 severely reduced the induction by estrogen and glucocorticoid. Likewise, point mutations of the protected guanine residues profoundly attenuated the response to these steroid hormones. In addition, in vitro binding activity correlated with in vivo functional activity. For example, mutant A4e shows no transcriptional activity in response to steroid hormones, and a corresponding oligomer does not bind protein in vitro. In contrast, mutant A4c is fully active in both contexts. These data support the contention that the ovalbumin gene is regulated by a steroid hormone-induced transcriptional cascade that culminates in the binding of chicken ovalbumin induced regulatory protein or protein complex (Chirp-I) to a DNA element from −891 to −878 in the SDRE.

Eukaryotic genes are primarily regulated by the modulation of the initiation of transcription via the binding of proteins to DNA flanking the coding sequences of genes. Two basic models have been proposed to explain the effects that *trans*-acting proteins have on transcription rates when bound to their *cis*-acting DNA elements (42). The first of these models involves altering the topology of the DNA by assembling a three-dimensional multimeric complex. The roles of several proteins that appear to be involved in rearranging chromatin structure are explained by this model. The second model invokes protein-protein interactions between the bound transcription factors and members of the general transcriptional machinery (42). These models are not mutually exclusive, and both rely on the ability of *trans*-acting factors to recognize a specific DNA element(s) in order to impart specificity to the response.

Steroid receptors apparently affect the initiation of transcription through both of the mechanisms described above, chromatin remodeling (3) and direct protein-protein interaction (17). Steroid hormones activate transcription by binding to specific receptor proteins, which then bind to well-defined DNA sequences in specific steroid-regulated genes (21). The steroid receptor binding sites or steroid response elements (SREs) behave as true enhancers. Many genes have SREs in their 5′-flanking DNA and are directly regulated by the binding of the steroid-receptor complex. The response of these genes is usually very rapid, within minutes, and requires no protein synthesis.

Some genes, however, take longer to respond to steroids and do not appear to directly bind the receptors. Genes that only respond to steroid hormones after a lag of several hours and require ongoing protein synthesis may be affected though a mechanism different from that of the primary-response or early-response genes. The secondary-response genes encode the vast majority of known steroid-responsive proteins (5). An intriguing model proposed to explain secondary-response genes is that of a transcriptional cascade (21). Landers and Spelsberg (21) proposed a model in which the primary-response genes code for transcription factors that are involved in the regulation of the secondary-response genes. This model is supported by the regulation of several proto-oncogene transcription factors (Fos, Jun, and Myc) by steroid hormones (10, 16, 31, 41). However, the model was originally proposed to explain the waves of polytene chromosome puffing in *Drosophila melanogaster*, and that system provides some of the best direct proof of gene regulation by transcriptional cascades (4). The transcription factor E74A is transcriptionally induced by 20-OH ecdysone (ecdysone) treatment (44). This factor subsequently regulates the transcription of secondary-response genes by directly binding to specific DNA elements (11, 44).

The gene that encodes the chicken egg white protein ovalbumin (Ov) is an attractive model system for studying secondary-response genes in vertebrates. The induction of this gene by four classes of steroid hormones does not appear to involve direct binding of steroid receptors and does require concomitant protein synthesis (24, 34, 35). Like other secondary-response genes, the synergistic induction of the Ov gene by estrogen and corticosterone follows a 2-h lag (24). The requisite addition of multiple steroid hormones, the 2-h lag time, and the requirement for functional protein synthesis imply that the induction of the Ov gene involves a novel mechanism requiring the new synthesis of a labile protein(s).

All steroidal induction of the Ov gene maps to a 120-bp region from −900 to −780, relative to the transcriptional start

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site, called the steroid-dependent regulatory element (SDRE) (36, 38). The SDRE does not function as an enhancer and requires the presence of another region from −308 to +9 to confer regulation by steroid hormones on a heterologous or homologous promoter (36). Neither element contains any consensus SREs (38). Thus, investigation has focused on determining how steroid hormones regulate the activity of this gene. The SDRE was extensively footprinted in vitro to examine whether steroid hormones affect the binding of proteins to this region. Three different cleavage reactions were used: DNase I, exonuclease III (Exo III), and dimethyl sulfate (DMS)-piperidine (27). The DNase I and Exo III footprinting defined a site between −900 and −800 that exhibited modest dependence on estrogen (27). Although not examined with respect to hormone dependence, methylation interference data defined interactions between guanine residues within the SDRE and oviduct nuclear proteins at −889, −885, −862, −842, −831, and −825 (27a, 39). Because footprinting analyses demonstrated that proteins bind contiguously to at least 100 bp of the SDRE, gel mobility shift analysis (GMSA) was used to define discrete sites. This analysis showed at least three regions (−900 to −853, −860 to −830, and −820 to −896) that were capable of independently binding nuclear proteins. However, only a twofold enhancement of binding to each of these sites was observed with estrogen treatment (27). This seems insufficient to account for the 20-fold induction of the Ov gene observed in vivo. However, protein synthesis inhibitors reduced binding to each of these three sites, suggesting that some components of the shifted complexes either are newly synthesized in response to steroid hormones or have a very short half-life (27). The site between −900 and −853 was of particular interest because all binding to it was abolished by inhibitors of protein synthesis.

The experiments in this study were designed to investigate further whether the labile protein complex that binds to the 5′ end of the SDRE is regulated by steroids. Linker-scanning mutations demonstrated that the region between −900 and −878 is essential for induction by steroid hormones. In vivo footprinting revealed that estrogen and corticosterone are absolutely required for the binding of a labile protein or protein complex, designated Chirp-I for chicken ovalbumin induced regulatory protein, to the region between −900 and −863. Point mutants were constructed based on the footprinting data to further delineate the binding site for Chirp-I and to examine the contributions that different bases make to the level of induction. Finally, GMSA with a functionally dead mutant, a functionally wild-type mutant, and the wild-type sequence demonstrated concordance between protein binding and transcriptional activation. A minimal site for Chirp-I from −891 to −878 is thus defined, with residues −889 and −885 being critical for binding. These data support the model that the Ov gene is induced by a transcriptional cascade initiated by steroid hormone-receptor complexes that culminates in the binding of Chirp-I to the SDRE.

MATERIALS AND METHODS

Plasmids. OvCAT-1.2 was constructed from OvCAT-1.35 as described for OvCAT-.900 (39). Linker-scanning mutants were created from OvCAT-1.2 by the method of Seal et al. (40). This method replaces specific nucleotides within a given stretch of DNA while maintaining the wild-type surrounding sequences and nucleotide spacing. In brief, oligonucleotides were synthesized to be used as primers in the PCR. Four primers were needed to produce each mutation: a primer corresponding to the 5′ end of the gene, a primer corresponding to the 3′ end of the gene, and a primer for each strand of DNA spanning the region to be mutated. These two internal primers were designed so that the 3′ end of each was homologous to wild-type Ov sequence, while the 5′ ends consisted of 10 bases of non-Ov sequence containing an *Xba*I site. These non-Ov sequences overlapped and defined the mutation. This construction minimizes the bases replaced by PCR-generated sequence to that between −1200 and −720, because the synthesized sequence was subcloned into a *Hin*dIII site in the vector and an endogenous *Bgl*II site at −720.

Point mutant plasmids were constructed by using a primer with a *Hin*dIII restriction site on the 5′ end and homology to Ov sequences between −900 and −876 except for the mutant bases. PCR was done between this primer and one outside of the SDRE homologous to Ov sequences −658 to −672. The plasmids were constructed by removing the sequences between −1200 and −720 from OvCAT-1.2 by using *Hin*dIII and *Bgl*II restriction sites and replacing that fragment with the PCR product containing the mutations. Thus, the final constructs are comparable to OvCAT-.900 because they contain Ov sequences between −900 and +9 except for the mutated bases (38). A wild-type plasmid was similarly constructed to use as a control.

Deletion mutants were similarly constructed; a PCR primer containing 25 bases of Ov sequence 3′ from the indicated nucleotide and flanked on the 5′ end with a *Bam*HI restriction enzyme site was used. For example, the −892 mutant was made by using a primer homologous to the Ov sequences from −892 to −876 with a *Bam*HI site on the 5′ end. PCR was done between this primer and one to the Ov sequences from −658 to −672. The PCR products were cloned into OvCAT-.864 (38) prepared by digestion with *Bam*HI and *Bgl*II. This generates a plasmid containing Ov sequences from the noted terminal base to +9 ligated upstream of the chloramphenicol acetyltransferase (CAT) gene.

All mutant sequences were confirmed by dideoxy sequencing with a United States Biochemicals kit according to the kit instructions.

Tubular gland cell culture. Tubular gland cells from sexually immature, estrogen-withdrawn chicken oviducts were isolated and transfected by $CaPO_4$ coprecipitation as previously described (36). Following transfection, all cells transfected with a given plasmid were pooled to reduce variations due to transfection efficiency. The linker-scanning and deletion mutants were cotransfected with RSVluciferase. All cells were plated into serum-free medium containing either insulin (50 ng/ml) or insulin plus estrogen (10^{-7} M) and corticosterone (10^{-6} M) and cultured for 24 to 36 h. The cells were harvested and lysed in Promega Multiple Assay Buffer using the manufacturer's suggested protocol.

In vivo footprinting. Cells cultured as described above were subsequently treated with cycloheximide where noted for various times. The medium was removed and replaced with fresh 37°C medium containing the appropriate hormones and 0.1% dimethyl sulfate (DMS). The cells were incubated for 2 min at room temperature, the medium was aspirated off, and the cells were washed three time with 1× phosphate-buffered saline (PBS). Footprints were generated by ligation-mediated (LM) PCR (13). The primers were made by Northern Biosciences, Inc. The gene-specific primers for the coding strand were: 1, (−658)-GCTCCATACAATGCAAACAATACTC-(−682); 2, (−710)-CTTGCAGTGTTCCTCAAAGATCTTCC-(−735); and 3, (−727)-GATCTTCCATGAATAACTTAATGGCCTGGC-(−756). The gene-specific primers for the noncoding strand were: 1, (−1080)-GTTTTCACAAAAGGAAGGAGAG-(−1059); 2, (−1052)-GAAAATGGCACTGACTAAACTTCAGC-(−1027); and 3, (−1046)-GGCACTGACTAAACTTCAGCTAGTGGTATAGG-(−1015).

Gels were quantified by scanning with a Bio-Rad densitometer and analyzed with Bio-Rad Molecular Analysis software.

Assays. CAT assays were performed by standard methods as previously described (39). Luciferase assays were done according to the manufacturer's instructions (Promega). Luciferase activity was measured with a Berthold Lumat LB9501 luminometer with 15-s measurement. All samples were measured in duplicate and repeated if the coefficient of variation exceeded 10%. CAT values for the point mutants were normalized to protein concentration as measured by the Bradford assay as previously described (39).

GMSA were performed with three double-stranded oligonucleotides: one homologous to the wild-type Ov SDRE sequence from −900 to −853 with *Hin*dIII overhanging ends, a second exactly the same as the first except that guanine residue −889 was changed to an adenine residue and guanine residue −885 was changed to a cytosine residue, and the third was the same as the second except that the base corresponding to −889 was a thymine residue and that corresponding to −885 was an adenine residue. These two mutant oligomers are the point mutants A4e and A4c, respectively, used in transfection studies. The oligonucleotides were labeled by Klenow fill-in reactions in the presence of radioactive dATP to a specific activity of approximately 10^8 cpm/μg. Gel shift binding assays were performed as previously described (27, 39). Competitions were done with either a 10- or 100-fold molar excess of the DNAs, as noted. The non-specific competitor was an oligonucleotides homologous to the pTZ18R polylinker region. Gels were run in Tris-glycine buffer as described elsewhere (7).

RESULTS

Linker-scanning mutants demonstrate that an element in the distal end of the SDRE is critical for induction by steroid hormones. The Ov gene represents a class of genes that do not directly respond to steroid hormones but rather are transcriptionally induced by intermediate proteins. Previous deletional analysis demonstrated an absolute requirement for the region of the SDRE from −892 to −864 for induction of OvCAT constructs by steroid hormones (27). Furthermore, in vitro

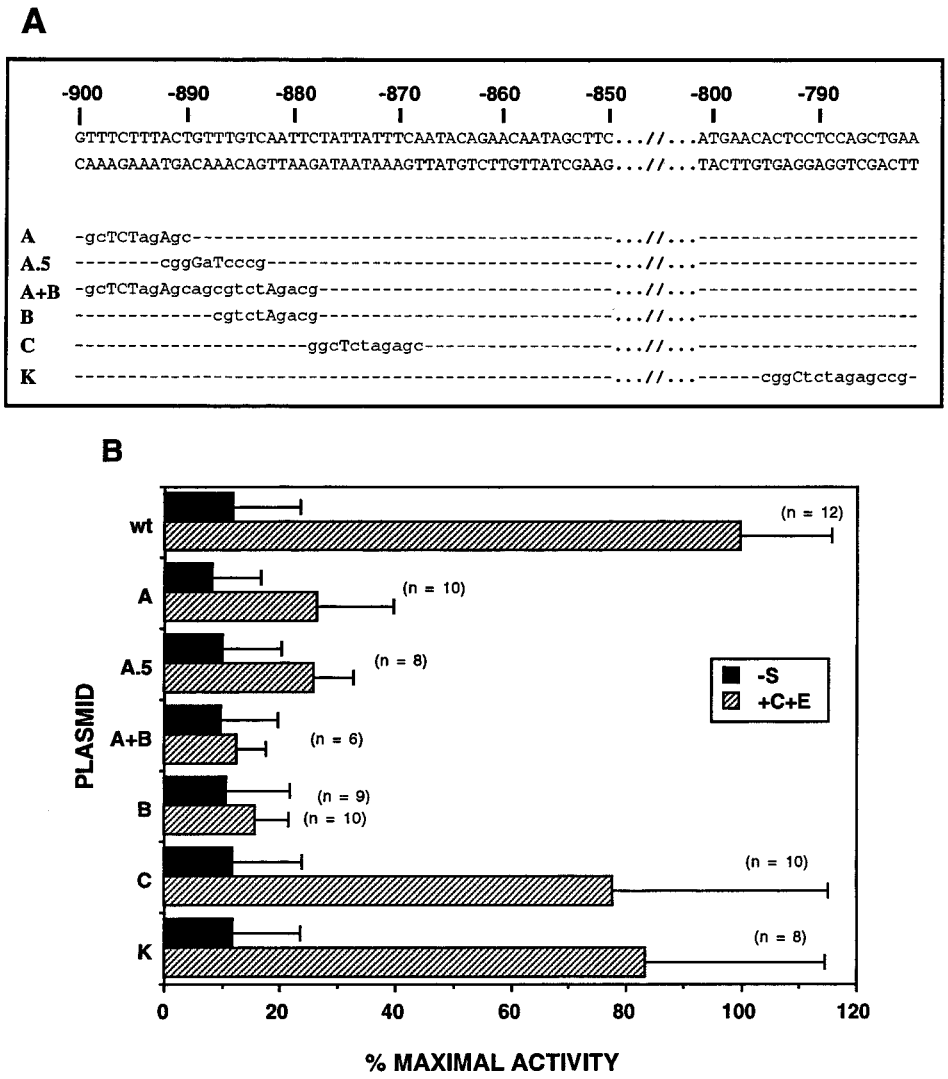

FIG. 1. Linker-scanning mutations demonstrate that the region between −900 and −878 is essential for induction by steroid hormones. (A) Sequences of the linker-scanning mutations. Bases homologous to the Ov sequence are capitalized or shown as dashes. Bases changed by the mutation are in lowercase letters. (B) Transcriptional activity as measured by CAT activity of the linker-scanning mutations. All constructs were transfected in duplicate for each treatment in at least three experiments. Transfected cells were subsequently cultured in serum-free medium containing insulin (50 ng/ml) alone (−S) or insulin plus corticosterone (10^{-6} M) and estrogen (10^{-7} M) (+C+E). Activity is normalized to that achieved with the wild-type (wt) OvCAT-1.2 construct in response to steroids. The CAT activity of the steroid-induced wild-type construct is typically 1 to 5% conversion. The total number of replicates is shown in parentheses.

binding assays demonstrated that a cycloheximide-sensitive protein(s) binds to the region from −900 to −853 in the SDRE (27). Additional GMSA limited that binding activity to the 5′ half of that region, from −900 to −876 (8b). In order to examine further the functional importance of this region, a series of linker-scanning mutations were made. These mutants retained all Ov sequences from −1200 to +9 except the indicated sequences, which were replaced with an *Xba*I site (Fig. 1A). The mutant constructs were cotransfected with RSVluciferase into primary cultured tubular gland cells by $CaPO_4$ coprecipitation. The transfected cells from a given DNA construct were pooled to reduce variations due to transfection efficiency prior to plating into medium containing either insulin (−S) or insulin, corticosterone, and estrogen (+C+E). Transcriptional activation was measured by the CAT assay and normalized to luciferase activity.

Notably, steroidal induction was reduced to 15 to 30% of wild-type induction in all mutants except mutants C and K (Fig. 1B). Because mutants A, A.5, A+B, and B all exhibited similar transcriptional activities, these mutants did not provide additional information about the 5′ boundary of the regulatory element. However, the mutation spanning the region from −878 to −869 (Fig. 1B, mutant C) was nearly wild type, implying that the 3′ border of this binding site is −878. When a sequence 3′ of the SDRE was altered, no significant change was observed (Fig. 1B, mutant K). Examination of the region between mutants C and K will be the subject of future work; however, mutation of the potential SRE half-site centered at −860 did not affect promoter activity significantly differently from other mutations in this region (17a). The data presented here indicate that a functional regulatory element that is essential for induction of the Ov gene by estrogen and corticosterone resides in the SDRE between −900 and −878.

The 5′ border of the DNA-binding site for Chirp-I is at −891. In order to determine the 5′ boundary of the Chirp-I binding site more precisely, transfections were performed with

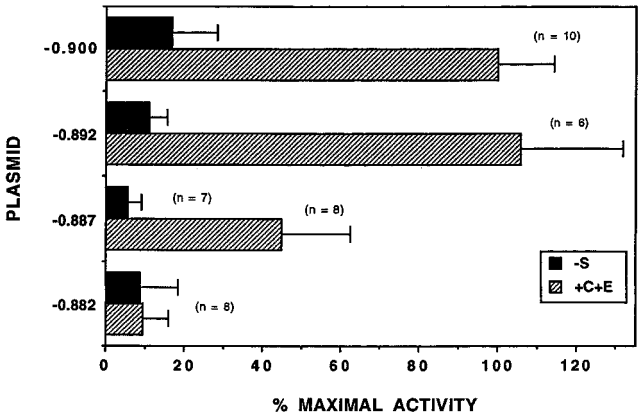

FIG. 2. −891 is the 5′ boundary of the Chirp-I binding site. 5′ deletion mutants were transfected into primary oviduct cell cultures as described in the legend to Fig. 1. Transcriptional activities of the deletion mutants were measured by CAT assay as described in the text. All constructs were transfected in duplicate for each treatment, and the data are the results of averaging at least three experiments. Transfection efficiency was normalized by using RSVluciferase. +C+E, cells grown with insulin, corticosterone, and estrogen; −S, cells grown with insulin alone. Results are compared with the activity of the wild-type OvCAT-.900 construct (−0.900) after treatment with both steroids. The total number of replicates is shown in parentheses.

5′ deletion mutants made by PCR mutagenesis. The deletion constructs contained all Ov sequences from the indicated base to +9. The control plasmid used for these transfection experiments was OvCAT-.900 (Fig. 2).

In agreement with previous results (27), deletion of sequences from −900 to −892 had no effect on induction by corticosterone and estrogen (Fig. 2). However, a deletion to −887 showed a 50% reduction in the response to steroid hormones, and a further deletion to −882 abolished steroidal induction. These data show that the 5′ boundary of the Chirp-I site is at −892 and that the region from −891 to −887 is required for maximal induction of the gene by steroid hormones. As will be demonstrated below, these data correlate well with the Chirp-I binding site identified by in vivo footprinting, since the adenine at −892 is hypersensitive to methylation, suggesting that it may flank the binding site.

In vivo footprinting shows that binding of Chirp-I is completely dependent on treatment with steroid hormones. Although GMSA had shown that binding of a protein(s) to the region between −900 and −853 was enhanced about twofold by estrogen, this slight augmentation of binding was not thought to be dramatic enough to explain the 20-fold induction of the Ov gene. Because, in a number of cases, hormone-dependent binding can be detected by in vivo but not by in vitro techniques (15, 25, 30), genomic footprinting with DMS was undertaken to ascertain whether convincing, steroid-inducible binding occurs within the cell. As a result of its size, DMS generates footprints that only depict guanine and adenine residues involved in very tight contacts with proteins. Primary oviduct tubular gland cells were cultured for 24 to 36 h in medium containing insulin (−S) or insulin, estrogen, and corticosterone (+S). Following culture, the medium was replaced with similar medium containing 0.1% DMS. The cells from similarly treated plates were pooled and lysed, and the isolated DNA was cleaved with piperidine. LM-PCR was performed on the samples with Ov-specific primers (13).

The resultant footprints of the region from −900 to −825 reveal several differences on the coding strand between cells treated with steroids and those that were untreated (Fig. 3A, B, and D; also see Fig. 4). No differences were observed on the noncoding strand (data not shown). Of particular interest, a steroid-dependent footprint on the coding strand between −892 and −862 appears to correlate with the binding of the protein or protein complex that we have designated Chirp-I (Fig. 3A). The tight interaction between Chirp-I and the DNA is indicated by the protection of guanine residues at −889 and −885. This is perhaps most apparent by computer analysis of densitometric scans (Fig. 3B and C). Fig. 3B represents individual scans of the three lanes in Fig. 3A, and the peaks on the scans are aligned to the bands in the gel. Although the overall intensity of the sample without steroids in this gel is slightly lower than that of the sample with steroids and the naked DNA sample, clear differences in the banding pattern are apparent. Most strikingly, the band at −885 in the samples without steroids and the naked DNA lane is missing from the sample with steroids. Computer analysis of similar results in other gels indicates that this guanine residue is protected 96% of the time in steroid-treated cells. This is confirmed by the data in Fig. 3C, which is a composite scan of all three lanes. In Fig. 3C the data for samples with steroids are shaded, and a clear gap in the shading occurs at −885, indicating that there is no band there. Guanine −889 in the Chirp-I binding site is also partially protected in the presence of steroids. Comparison of several gels shows that this residue is protected about 65% of the time in cells that were treated with estrogen and corticosterone. Thus, steroid hormone treatment of primary oviduct cells elicits the tight association of the Chirp-I protein or protein complex with guanines −889 and −885.

Unfortunately, no other guanine residues reside in the coding strand between −900 and −862, so it is difficult to ascertain with this technique which other bases in the Chirp-I binding site are involved in steroid-dependent binding. However, the boundaries of the Chirp-I site are inferred from the hypersensitive adenine residues at −892 and −865 (Fig. 3D and E). Although these hypersensitivities are not readily apparent on the exposure of the gel shown here, we have consistently seen them on gels exposed for a longer time (see Fig. 4). Methyladenine residues are not quantitatively cleaved by piperidine treatment. Therefore, the presence of these adenine residues is indicative of extreme hypersensitivity. In fact, computer analysis of several gels indicates that they are fivefold more hypersensitive in the steroid-treated cells than in the untreated cells.

To obtain a more graphic and quantitative analysis of the footprinting data, the area under each peak in Fig. 3B was determined by computer analysis. As an internal control, each band within the steroid-treated and untreated lanes was compared with the respective −910 band. The −910 band was chosen because it lies outside the region previously shown to be functionally relevant (38) and because its intensity is consistently hormone independent. These data were plotted against the band position (Fig. 3D and E); Fig. 3E is just an expanded view of the region from −892 to −885. Steroid-dependent protections are evident by points where the ratios with steroids are lower than the ratios without steroids. Hypersensitivities are, of course, the opposite. The steroid-dependent protection of bands −889, −885, −862, and −842 is quite evident, especially compared with the numerous bands that show no steroid-dependent change, such as those at −910, −900, −854, and −831.

Interestingly, when the scan was closely examined, the region between −900 and −830 appears to contain binding sites for at least two proteins or protein complexes. The second site appears to be located between about −863 and −825, with a steroid-dependent 80% protection of guanine residue −842 (see Fig. 3A, C, and E). This is supported by previous in vitro

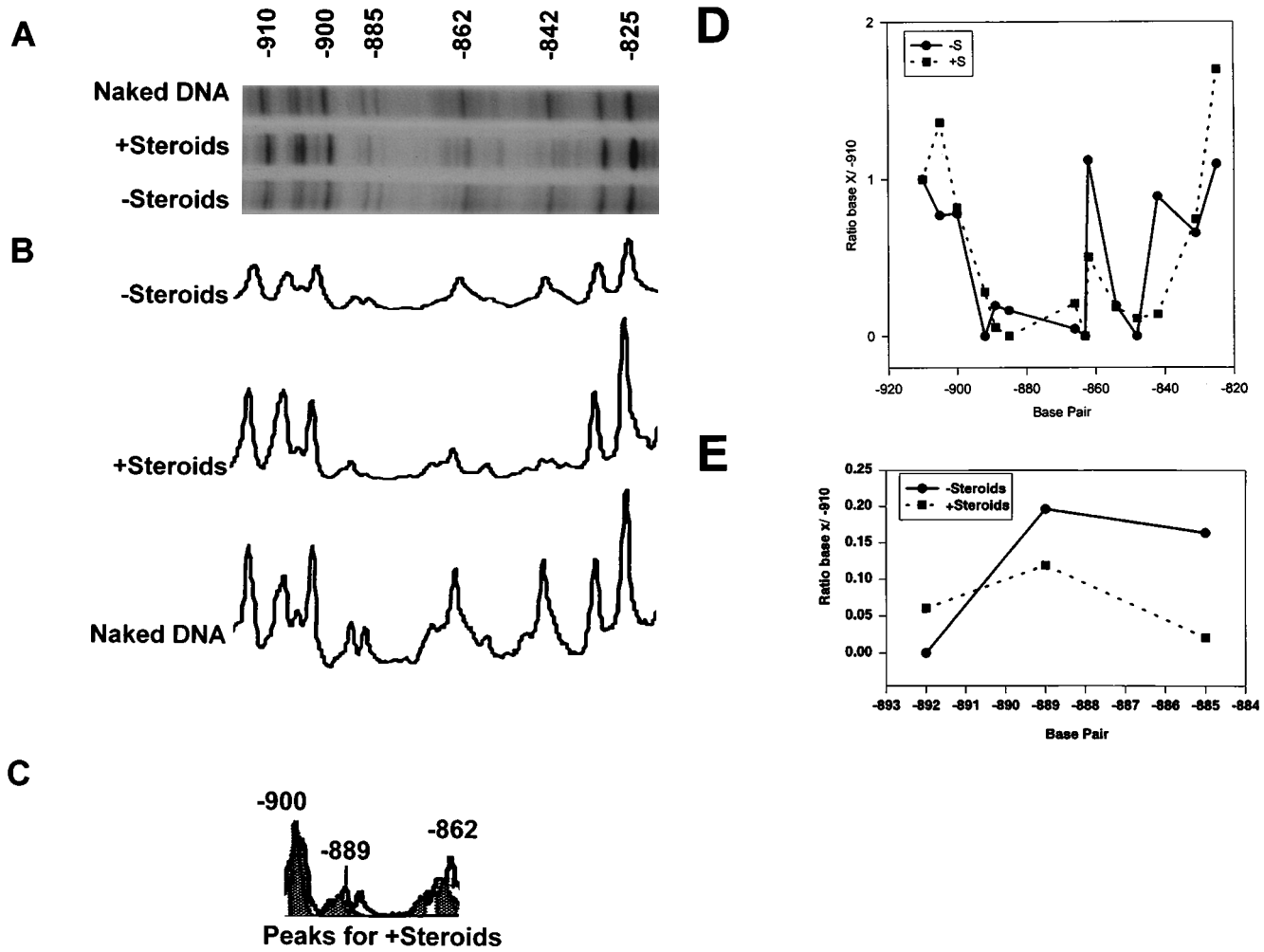

FIG. 3. In vivo footprinting reveals a steroid-specific binding site centered at −887. (A) In vivo footprinting gel. DNA was extracted from cells that had been cultured with insulin, estrogen, and corticosterone (+Steroids) or with insulin alone (−Steroids) and then subjected to DMS treatment in vivo. Ov-specific primers were used to amplify the DNA by LM-PCR as described in Materials and Methods. As a control to identify guanine residues, DNA stripped of proteins was treated with DMS in vitro (naked DNA). The nucleotides corresponding to the bands in the naked DNA are identified respective to the transcriptional start site. (B) Scans with a Bio-Rad densitometer of each of the lanes in the gel shown in panel A. The scans are aligned with the gel above so that peaks correspond to bands. (C) Molecular Analyst peak assignment to the bands in the +Steroids lane in the region between −900 and −862. The scans of the other two lanes are still present but are mostly obscured by the shaded peaks except at −885, where the residue is completely protected in the +Steroids lane. (D) Graph of the ratio between the area of the peak corresponding to band −910 and each other band in the −Steroids and the +Steroids lanes in the region between −910 and −825. (E) Same as panel D except expanded to show details in the region between −892 and −885.

GMSA data (27). To summarize, these genomic footprinting data provide evidence that the estrogen- and corticosterone-dependent binding of Chirp-I is to an element centered around residue −887 and implicate the binding of another protein or protein complex to the region from −863 to −825. This is the first observation of the binding of a steroid-dependent protein to a functionally relevant region of the Ov gene after many years of searching.

In vivo footprinting reveals that Chirp-I contains a labile protein. Previous results have shown that a labile protein or protein complex binds to the SDRE between −900 and −853 (27). To determine whether the steroid-dependent binding seen in vivo was attributable to the same labile protein, cycloheximide studies were undertaken. Cells cultured as described above were subsequently treated with cycloheximide for either 1.5 or 3 h. The cells were then treated with DMS, and the DNA was harvested and subjected to LM-PCR (13).

Again, treatment with steroid hormones elicits the complete protection of guanine residue −885 and the partial protection of guanine residue −889 as well as the hypersensitivity of adenine residues −892 and −865 (Fig. 4, compare lanes 2 and 3 with lanes 6 and 7). Subsequent exposure of steroid-treated cells to cycloheximide for 1.5 h resulted in a partial reversal of the steroid hormone-induced changes; the hypersensitivity of the adenine residues and the partial protection of guanine residue −889 are abolished, while guanine residue −885 is only partially protected (Fig. 4, compare lanes 2 and 3 with lane 4). Following a 3-h treatment with cycloheximide, the steroid-treated cells are indistinguishable from the untreated cells (Fig. 4, compare lane 5 with lanes 6 and 7). These data support the contention that steroids are acting to induce the synthesis of a labile transcription factor.

Point-mutational analysis demonstrates that guanine residues −889 and −885 are critical for the binding of Chirp-I. In vivo footprinting revealed that guanines −889 and −885 are protected by Chirp-I after treatment with estrogen and corti-

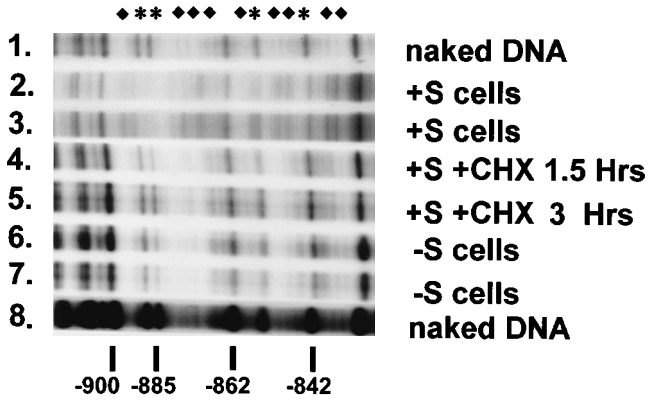

FIG. 4. In vivo footprinting of cycloheximide-treated cells unveils the lability of Chirp-I. Lanes 1 and 8, naked DNA treated in vitro with DMS; lanes 2 and 3, DMS-treated cells cultured with insulin, estrogen, and corticosterone (+S); lane 4, cells cultured with insulin, estrogen, and corticosterone and then treated with cycloheximide (CHX) for 1.5 h before DMS treatment; lane 5, same as lane 4 except cycloheximide treatment was for 3 h; lanes 6 and 7, cells grown with insulin alone (−S) and then treated with DMS. Bands are numbered relative to the Ov gene transcriptional start site. Comparison of the +S lanes with either the naked DNA or the −S lanes shows alterations due to steroid treatment: steroid hormone-dependent hypersensitivity is denoted by solid diamonds, and protection is denoted by asterisks.

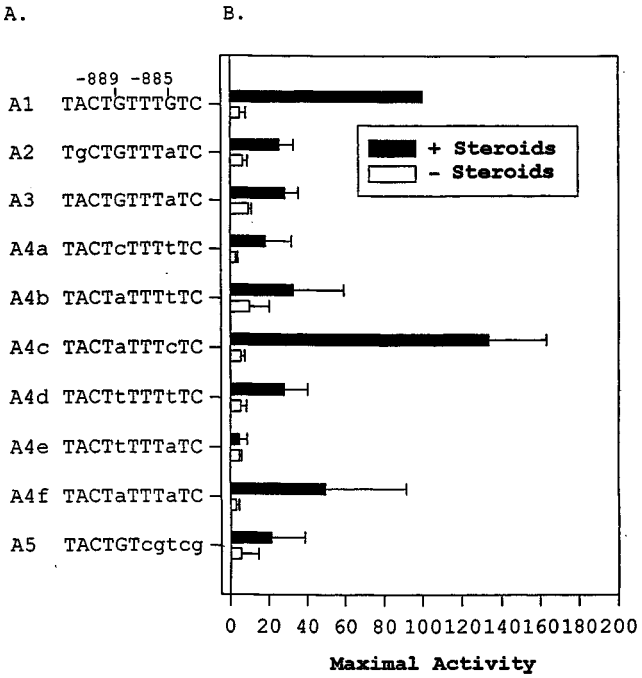

FIG. 5. Guanines −889 and −885 are essential for the induction by steroid hormones. (A) Sequences of the point mutation constructs between −893 and −883. All Ov sequence was retained from −900 to +9 except for the mutations shown in lowercase letters. (B) Plasmids containing the indicated point mutations were transfected into primary oviduct cells as described in the legend to Fig. 1. Transcriptional activity was measured by CAT assay. +Steroids, cells grown with insulin, estrogen and corticosterone; −steroids, cells grown with insulin alone. The data represented here are the averages of three independent experiments done in duplicate for each treatment (six replicates for each treatment). The data are normalized to the activity of the wild-type construct (A1) in the presence of steroid hormones and are given as percentages of maximal activity.

costerone. To confirm that those bases are functionally relevant for the induction by steroids, point mutagenesis was performed. OvCAT constructs containing all of the Ov sequences from −900 to +9 were made by PCR mutagenesis, with single, double, or multiple mutations (Fig. 5A). These constructs were transfected into primary oviduct tubular gland cells by $CaPO_4$ coprecipitation. The transcriptional activity was measured by CAT activity as previously described (39).

Mutation of guanine residue −885 to an adenine residue results in at least a 60% loss of transcriptional activity in response to estrogen and corticosterone (Fig. 5B, mutation A3). Double mutation of −885 guanine and either the −892 adenine or the −889 guanine attenuated the induction by steroids by 50 to 95% (Fig. 5B, mutations A2-A4b and A4d-A5) except for mutation A4c. In that case, when G(−889) was converted to an adenine residue and G(−885) was converted to a cytosine residue, a slightly higher than wild type response was consistently observed (Fig. 5B, mutation A4c). The most striking effect was seen with mutation A4e, when G(−889) was mutated to a thymine residue and G(−885) was mutated to an adenine. The transcriptional induction by steroid hormones of this construct is completely abolished. These data indicate that the protected guanine residues −889 and −885 are critical for the binding of Chirp-I. However, some base substitutions are more deleterious than others (Fig. 5B, mutant A4e versus A2, A3, A4a-d, and A4f). Interestingly, it is possible to make a double substitution that restores wild-type activity (Fig. 5B, mutant A4c compared with wild-type A1). This suggests that other residues in the binding site also make tight contacts with Chirp-I.

Protein binding to the Chirp-I element in vitro correlates with functional activity in vivo. Functionally important transcription factors frequently bind to their DNA recognition sequences in vitro (26). Therefore, as a final confirmation of the Chirp-I binding site, GMSA was done. Previously, this region was shown to bind a cycloheximide-sensitive protein or protein complex (27). Two of the double point mutations were of particular interest: A4e because it was unable to respond to steroid treatment, and A4c because it had wild-type transcriptional activity. Oligonucleotides homologous to the wild-type Ov sequence from −900 to −853 and both the A4e [G(−889) to T and G(−885) to A] and the A4c [G(−889) to A and G(−885) to C] double mutants were synthesized. The oligonucleotides were labeled by Klenow fill-in reactions and incubated with laying-hen oviduct nuclear proteins as previously described (27). Cross competitions were done by simultaneous addition of unlabeled oligomers at the molar ratios noted in Fig. 6.

The binding of Chirp-I to the wild-type probe generates two major bands (Fig. 6A, lane 2, specific bands marked with arrowheads). The specificity was determined by self-competition with a 10- or 100-fold molar excess (Fig. 6A, lanes 3 and 4, respectively) and by the lack of competition with a heterologous DNA at a 100-fold molar excess (Fig. 6A, lane 7). The A4e mutant oligonucleotide does not compete with the wild type (Fig. 6A, lanes 5 and 6) at either a 10- or 100-fold molar excess. Likewise, Chirp-I does not bind to the mutant A4e oligomer (Fig. 6A, lane 9). The limited binding to the A4e mutant oligomer appears to be nonspecific (Fig. 6A, lanes 10 to 14). Therefore, these data demonstrate that the lack of induction of the A4e construct can be ascribed to its inability to bind Chirp-I, corroborating the functional data.

Interestingly, the A4c mutant sequence appears to bind Chirp-I at least as well as the wild-type sequence does (Fig. 6B, compare lanes 5 and 6). The same two major bands are apparent with the A4c mutant oligomer as with the wild-type probe. Furthermore, the wild-type oligomer does not appear to compete as well as the A4c oligomer for the binding of Chirp-I

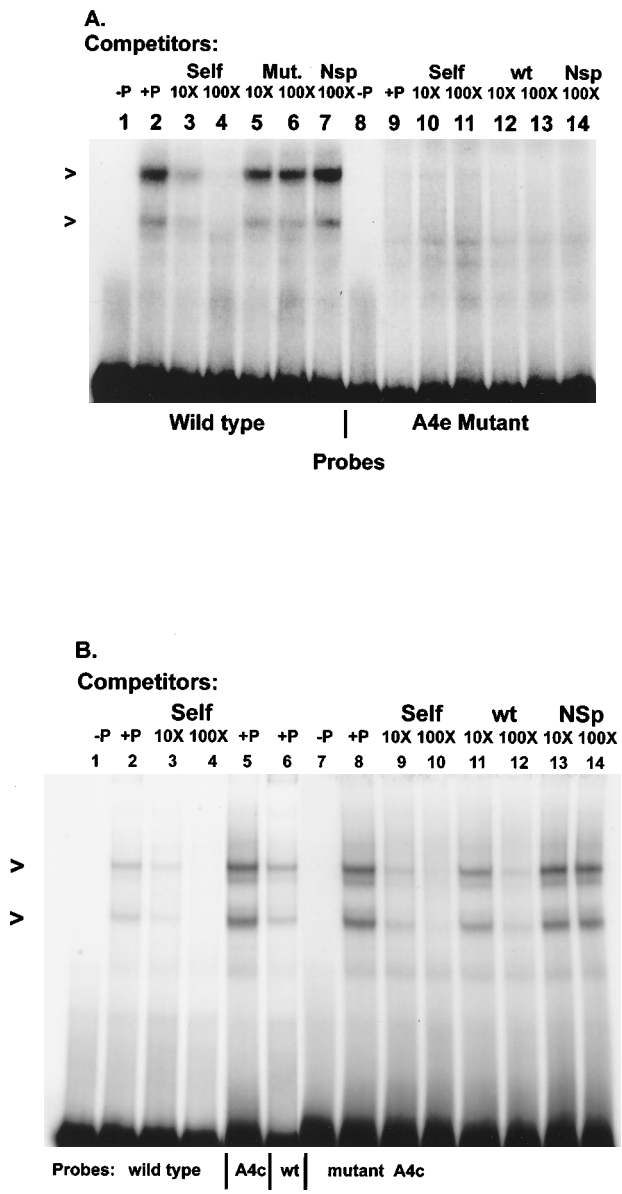

FIG. 6. GMSA corroborates the point mutational analysis. (A) Comparison of protein binding to the A4e mutant with that to the wild type (wt). Lanes 1 and 8, probe (P) alone; lanes 2 to 7, shifted complexes created when a probe homologous to the wild-type SDRE (−900 to −853) was bound by laying-hen nuclear protein extracts (wild type); lanes 9 to 14, result of using an oligonucleotide homologous to the A4e mutation as the probe with the same laying-hen nuclear extracts. Competitions were done by the concurrent addition of a nonradioactive homologous oligomer (self; lanes 3, 4, 10, and 11), A4e mutant oligomer (Mut.; lanes 5 and 6), wild-type oligomer (wt; lanes 12 and 13), or a nonspecific oligomer (Nsp; lanes 7 and 14). The nonspecific competitor was an oligonucleotide homologous to the polylinker of pTZ18R. (B) Comparison of protein binding to the A4c mutant with that of the wild type (wt). Lanes 1 and 7, probe (P) alone. All other lanes represent incubations done with laying-hen oviduct nuclear proteins. Lanes 2 to 4 and 6 show the shifted complexes with the wild-type oligonucleotide as a probe. Lanes 5 and 8 to 14 show the shifted complexes from using an oligonucleotide homologous to the A4c mutation as the probe. The designations for competitors are the same as in panel A. Specific bands generated by binding of Chirp-I to the probe are marked by arrowheads.

(Fig. 6B, compare lanes 9 and 10 with 11 and 12). Thus, all the GMSA data strongly support the functional data. The A4c mutant shows a slightly greater than wild type transcription rate, and this sequence shows a slightly greater binding of the Chirp-I complex than the wild-type sequence. Likewise, the A4e mutant did not support any *trans* activation and is incapable of binding Chirp-I.

DISCUSSION

Many genes were classified as secondary-response genes based on the original definition of a transcriptional response to steroid hormones that was both sensitive to inhibitors of protein synthesis and delayed. Among these putative secondary-response genes are the genes for rat arginase, α_1-acid glycoprotein, rat tryptophan oxygenase, and rat $\alpha_{2\mu}$-globulin (1, 8, 14, 20). Although these genes were classified as secondary-response genes many years ago, very little work has been done to further characterize the important factors involved in this novel response to steroid hormones. The best characterized is the rat arginase gene. A 233-bp region from intron 7 of the rat arginase gene may be involved in the secondary response to glucocorticoid treatment (14). This region binds four proteins or protein complexes, two of which appear to be similar to each other by cross competition. Both of these are C/EBP family members (14). The arginase enhancer mediates the glucocorticoid response in a cell-specific manner, but no attempt was made to determine whether the binding of any of the proteins to this 233-bp fragment was sensitive to cycloheximide (14). Similarly, a regulatory region from the $\alpha_{2\mu}$-globin gene between −237 and −102 acts to confer glucocorticoid responsiveness but does not bind cycloheximide-sensitive proteins (1). Although the α_1-acid glycoprotein gene is a classical example of a secondary-response gene, it probably does not actually fall in that classification, as it appears to respond to glucocorticoid treatment directly through a consensus glucocorticoid response element located between −121 and −107 (20). The cycloheximide sensitivity of this gene is conferred by another region between −106 and −42, although no further characterization of that site has been done (20). Hundreds of other genes are estimated to be regulated by steroid hormones through secondary-response pathways (5), yet this remains an area with very little active research. The Ov gene thus represents an excellent model system for furthering the understanding of steroid-mediated secondary-response mechanisms in vertebrates.

Herein, we have shown that a protein or protein complex, designated Chirp-I, involved in the expression of the Ov gene binds to a region from −891 to −878 (Fig. 7). Furthermore, certain bases within this binding site are critical for transcriptional activity and for binding activity (Fig. 6 and 7). The binding of Chirp-I in vivo requires treatment with steroid hormones (Fig. 3 and 7) and ongoing protein synthesis (Fig. 4, lanes 4 and 5, and Fig. 7). These data support the hypothesis that Chirp-I, or a member of this complex, is newly synthesized in response to treatment with steroid hormones.

Although other explanations are possible, they are more complicated. For example, Chirp-I might be modified by a protein that is synthesized in response to steroid hormones. Alternatively, the steroid-dependent protein might be an accessory factor that does not directly bind to the DNA but somehow stabilizes the binding of Chirp-I. Each of these alternatives invokes an additional protein's being involved in the response to steroids. Without further evidence, the simpler model is favored. Cloning of the Chirp-I gene is under way, and its characterization will determine whether it is a primary-response gene.

Understanding the mechanisms by which steroid hormones induce the Ov gene has been refractory to in vitro analysis. Although transcription of the gene increases approximately 20-fold in response to estrogen and corticosterone treatment,

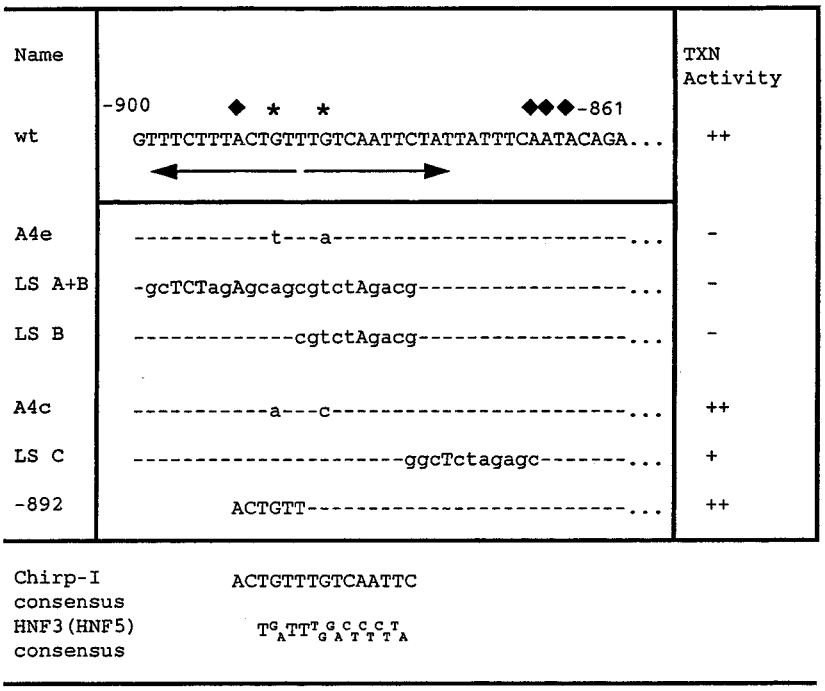

FIG. 7. Summary of results. For transcriptional (TXN) activity results, see Fig. 1, 4, and 5. The HNF3 (HNF5) consensus sequence is from reference 24. For details on other data, see the text. *, decrease in DMS sensitivity; ◆, increase in DMS sensitivity. LS, linker-scanning mutant.

GMSA and in vitro DNase I footprinting revealed changes of only about twofold in protein binding to the SDRE (27). The discrepancy between the in vivo footprinting data and the in vitro data is thus of some concern. One possible explanation is that a completely different protein is being assayed by the two approaches. However, this is hard to reconcile with the observation that, both in vitro and in vivo, binding is abolished by cycloheximide treatment. Sensitivity to cycloheximide is of functional importance because the Ov gene becomes transcriptionally silent within 3 h of treating hens or cultured cells with it (27). Furthermore, the correlation of the transcriptional activity of the point mutants with their binding activity in GMSA suggests that the proteins being assayed in vitro are at least part of the functional complex that is active within the cell. Therefore, another possible explanation is that Chirp-I is actually a protein complex and the DNA-binding protein is not the protein that is induced by steroids. The larger complex may not be stable in the GMSA. Labs working on other steroid-dependent genes have had similar problems (6, 9, 37). For example, the mouse sex-limited protein shows no sex-dependent in vitro DNase I footprint in a region that is required for response to androgen treatment (2). However, in vivo, a distinct footprint is visible only in males or androgen-treated females (37). Therefore, as in our system, the genomic footprinting and functional data are in accordance.

The Chirp-I binding site forms the core of a nearly perfect mirror repeat of 12 bp with a central thymine residue at −887 (Fig. 7). Although the bases on either side of the central T are identical, they are structurally distinguished by polarity differences. Therefore, this unusual repeat is of unknown biological significance. The Chirp-I binding site has no homology to the binding sites of any known transcription factors except HNF3 (Fig. 7) (32). Members of the HNF3 family of transcription factors bind to DNA rather promiscuously, since there are several nonspecific bases in the motif (28, 32). Although there are two mismatches between the consensus HNF3 element and the Chirp-I site, it is possible that Chirp-I is or contains a member of this superfamily of transcription factors. However, preliminary GMSA data suggest that Chirp-I is not identical to HNF3 (8a).

Chromatin structure studies have defined two classes of transcriptionally active genes. Those that have poised basal machinery are called preset. Several genes in this class, such as c-*jun*, that have fully assembled enhancers that need only a single transducing signal to become transcriptionally active have been investigated (33). Another example is the *Drosophila hsp26* gene. The chromatin arrangement of this gene is established during or immediately following DNA replication by the interaction of GAGA factor with CT repeat elements in its flanking DNA (23).

At the opposite end of the spectrum, remodeling genes that load transcription factors only after a signal has been received have been described. Several steroid-regulated genes, including the rat tyrosine aminotransferase gene, the chicken vitellogenin genes, and the mouse mammary tumor virus long terminal repeat, are remodeling genes. In these cases, the steroid-receptor complex interacts with an SRE to elicit the binding of cell-specific and ubiquitous transcription factors in vivo, presumably by altering the arrangement of nucleosomes in the vicinity of the SRE (3, 6, 15, 22, 30, 43). The best studied of these is the mouse mammary tumor virus promoter, which clearly demonstrates the rearrangement of a nucleosome after treatment with glucocorticoid (3). The remodeled chromatin can bind NF-1 and is thereby rendered transcriptionally active.

It is difficult to hypothesize what role Chirp-I plays in the activation of the Ov gene by steroid hormones. Many of the necessary interactions are still very poorly understood. The Ov gene appears to be a remodeling gene, as it has an estrogen-inducible hypersensitive site in the region spanning −1030 to −650 (19) and as steroids induce protein binding to its flanking

DNA (Fig. 3 and 4). Although treatment with estrogen results in rearranged chromatin, it does not involve an interaction between the estrogen receptor and a typical estrogen response element within the SDRE (38). From the data presented herein, the more probable explanation is that steroids are acting via Chirp-I to elicit this change in chromatin structure. Interestingly, HNF3, the only known transcription factor with a binding site homologous to the Chirp-I binding site, has been implicated in nucleosome disruption of the albumin promoter (25). Therefore, it is possible that Chirp-I affects the transcription of the Ov gene by both of the above models. Chirp-I may alter the topology of the surrounding chromatin, giving rise to an inducible DNase I-hypersensitive site. In addition, since its binding site is required for full induction of transiently transfected OvCAT constructs, it must also enhance the loading of basal machinery, presumably through a protein-protein interaction(s). Experiments are in progress to directly test this.

Another estrogen-inducible DNase I-hypersensitive site at −3100 (19) was shown to function as an estrogen-dependent enhancer when cloned abutting a minimal (−58 to +1) Ov promoter (18). However, this work was done in HeLa cells cotransfected with an estrogen receptor expression vector, and this region has no effect on the induction of OvCAT constructs by steroid hormones in oviduct cells (27, 30, 36, 38). Thus, we speculate that the distal estrogen-dependent hypersensitive site is involved in establishing an open chromatin arrangement over the Ov gene during the initial differentiation of the oviduct in response to estrogen. This upstream element probably plays no role in the subsequent secondary induction of the Ov gene by estrogen.

Chirp-I is the first example of a steroid-dependent protein involved in the regulation of the transcription of a secondary-response gene in a vertebrate system. Although the model of transcriptional cascades was proposed to explain the steriodal induction of secondary-response genes (21), no primary-response gene has ever been shown to be involved in the regulation of a secondary response gene except in *Drosophila* cells (11, 44). Although the chicken Ov gene was one of the first genes ever cloned (12) and although it has long served as a model for investigating how estrogen increases gene expression, the relevant molecular mechanisms have remained elusive (34). Finally, a piece of that puzzle is revealed by the definition of the binding site for Chirp-I, a steroid-responsive labile protein or protein complex.

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