A Far Upstream Ovalbumin Enhancer Binds Nuclear Factor-1-like Factor*

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Band-shifting and DNase I footprinting analyses detected a specific DNA binding protein extracted from oviduct nuclei that binds to the ovalbumin gene 5' sequence between -1094 and -1125. This -1100 fragment, when inserted upstream of the SV40 or ovalbumin promoters fused to a chloramphenicol acetyltransferase reporter gene, enhances chloramphenicol acetyltransferase activity 6-10-fold following transfection into CV1 cells. The sequence to which the oviduct factor binds contains a nuclear factor-1 (NF-1) half-site (GCCAA). An oligonucleotide matching the sequence of the adenosine NF-1 binding site competed for binding to the -1100 footprinted region with a higher affinity than an oligonucleotide for the -1100 region itself. Similarly, the -1100 region oligonucleotide also competes for binding of the factor to the NF-1 oligonucleotide. These data suggest that the oviduct factor which binds to the -1100 region is an NF-1-like protein that serves as a steroid hormone-independent enhancer of the ovalbumin gene transcription.

Interactions between specific DNA sequences and regulatory protein factors play important roles in the control of eucaryotic gene expression. Several cis-acting DNA regulatory elements and their respective trans-acting proteins have been identified. Most genes transcribed by RNA polymerase II contain a TATA box 25-30 bp 5' of the transcription start site; this element is required for accurate and efficient initiation of transcription (Grosschedl and Birnstiel, 1980). A protein factor that specifically interacts with the TATA box has been identified in Drosophila and in human cells (Parker and Topol, 1984; Sawadogo and Roeder, 1985). Upstream of the TATA box, many eucaryotic genes contain the sequence CCAAT (Grosveld et al., 1982; Benoist et al., 1986). Nuclear proteins that bind to this sequence have recently been identified in human and in rodent cells (Jones et al., 1985; Graves et al., 1986; Cohen et al., 1986). Recent work suggests that there is a family of CCAAT box-binding proteins (Dorn et al., 1987; Oikarinen et al., 1987). One protein of the family, the CCAAT box-binding transcription factor (CTF) from HeLa cells, has been purified to apparent homogeneity and appears to be identical or very similar to the protein nuclear factor-1 (NF-1) (Jones et al., 1987; Rosenfeld and Kelly, 1986). NF-1 was originally identified as a cellular protein required for adenovirus replication (Nagata et al., 1983; Hay, 1985). NF-1 binding sequences in the MMTV long terminal repeat are known to enhance transcription (Miksicke et al., 1987).

In addition to the promoter elements, another set of cis-acting regulatory sequences have been identified as enhancers. Enhancers are defined by their ability to activate transcription of linked genes in a fashion relatively independent of orientation and position (Banerji et al., 1981; Moreau et al., 1981). First identified in SV40 and polyomavirus (Banerji et al., 1981; Devilleers and Schaffner, 1981), enhancers and the trans-acting factors that bind to them have been identified for several cellular genes (see review, Serfling et al., 1985; Maniatis et al., 1987).

For the steroid-regulated chicken ovalbumin gene, several cis- and trans-acting factors have been identified. A TATA box in the proximal promoter region is necessary for efficient initiation (Tsai et al., 1981a). The chicken ovalbumin upstream promoter (COUP) element, which is essential for accurate transcription in vivo and in vitro, is located approximately 90 bp upstream of the initiation start site (Knoll et al., 1983; Pastorcic et al., 1986). This sequence interacts with the COUP transcription factor, which has been purified from HeLa cell and oviduct extracts (Sagami et al., 1986; Wang et al., 1987; Bagchi et al., 1987). Additionally, several sequences further upstream specifically bind factors from oviduct nuclear extract. The roles of these binding factors in ovalbumin gene expression are currently under investigation.

In this paper, a sequence located approximately -1100 bp upstream of the transcription start site is shown to act in transient transfection assays as a transcriptional enhancer for the SV40 and ovalbumin promoters. Band-shifting competition assays show that a factor binding to this sequence is related to NF-1.

**EXPERIMENTAL PROCEDURES**

**Band-shifting Assay**—A plasmid pOV1.7, containing 1.4 kilobases of the ovalbumin 5'-flanking sequence (Roop et al., 1978; Tsai et al., 1981a) was digested with Accl, end-labeled with [32P]dCTP (3000 Ci/mmol; ICN Pharmaceuticals, Inc.) and digested by RsaI. The 220-bp end-labeled fragment or kinase-labeled oligonucleotides (0.3-1.0 ng, 10⁵ cpm) were incubated with the DE175 fraction of oviduct nuclear extract (Tsai et al., 1981b). Reactions contained excess unlabeled poly(dT-dC), 10 mM HEPES, 2.5 mM MgCl₂, 10% glycerol, 100 mM KCl, 0.2 mM EDTA, and 2 mM dithiothreitol (Sagami et al., 1986). Protein-DNA complexes formed at room temperature were separated from protein-free DNA by electrophoresis in native polyacrylamide gels (Fried and Crothers, 1981). In competition assays, identical conditions were used after mixing the appropriate molar amounts of unlabeled competitor DNA with the probe before adding the protein.

DNase I Footprinting—DNase I footprinting was performed as described by Galas and Schmitz (1978) and modified by Sagami et al.

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AccI-RsaI fragment of the ovalbumin promoter, extending from -953 to -1174 ("-1100") fragment. The reactions were incubated with oviduct nuclear extract DE176 fraction at room temperature for 10 min. Pancreatic DNase I ( Worthington) was then added, and the mixture was incubated at 37 °C for 30 s. The reaction was stopped by adding 100 μl of 10 mM EDTA, 0.1% sodium dodecyl sulfate, 50 μg of Proteinase K, 1.5 μg of pBR322 and incubating at 37 °C for 15 min. Samples were extracted with phenol/chloroform, ethanol-precipitated, heated to 90 °C, and separated on 8% polyacrylamide, 5 μm sequencing gels.

Plasmids Used for Transfections—The 220-bp AccI-Real (-1100) fragment of pOV17 was rendered blunt-ended with Klenow enzyme and ligated to BglII linkers. This fragment was inserted into one of the following vectors (Fig. 2): 1) pAloCAT; 2) pBLCAT2 (Laimins et al., 1982), which contains the SV40 early promoter with the enhancer deleted, fused to the chloramphenicol acetyltransferase (CAT) gene and the SV40 poly(A) signal; the -1100 fragment or a synthetic oligonucleotide representing only the DNase I-protected region of this fragment was inserted into the BglII site adjacent to the promoter; 2) pBLCAT2 (Lockow and Schütz, 1987), contains the Herpes simplex virus tk promoter fused to the CAT gene; 3) the -1100 fragment was inserted into the BamHI site located in the polylinker adjacent to the tk promoter; 3) pOVCATMA, which contains the BglII-TaqI (converted to HindIII) fragment of the ovalbumin promoter (+41 to -723); pAloCAT, was digested with BglII and HindIII, and the entire SV40 promoter was replaced by this ovalbumin promoter fragment; the -1100 fragment was inserted at the BglII site.

Expression vectors of steroid hormone receptor genes used in cotransfection experiments were: 1) p79K (Conneely et al., 1987) containing 3.1 kilobases of the chicken progesterone receptor cDNA cloned into the EcoRI site of the p91023 expression vector (Wong et al., 1985); and 2) pHER-91023 containing the large open reading frame of the human estrogen receptor cDNA (Walter et al., 1985). To interrupt the open reading frames near the 5' end of this cDNA, sequences upstream of the TφI site were deleted, and this site was converted to BamHI. EcoRI linkers were added, and the resulting BamHI-EcoRI fragment was inserted into the EcoRI site of p91023 to create pHER-91023.

Cell Transfections—CVI cells were grown in 10-cm dishes at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The medium was supplemented with 10 °M each of estrogen and progesterone for cotransfection experiments. 5-10 μg of DNA were introduced into 40-70% confluent cells in serum-free media using Polybrene (5 μg/ml) (Kawai and Nishizawa, 1984). 18 h post-transfection, the cell extracts (25-100 μg) of soluble protein were assayed for CAT activity overnight by the method of Gorman et al. (1982).

RESULTS

Delineation of the Site of Oviduct Factor Interaction—An AccI-Real fragment of the ovalbumin promoter, extending from -953 to -1174 (-1100 fragment), specifically interacts with a factor in the DE175 fraction of oviduct nuclear extracts of hormonally stimulated chicks. The site of interaction was delineated by DNase I footprinting. Fig. 1 shows the footprint on both strands and the sequences protected by the factor(s) in the DE175 fraction. The protected region on the top strand extended from approximately -1096 to -1125 and on the bottom strand from approximately -1084 to -1125. There was a strong hypersenstive site on both strands at position -1103. In addition, the bottom strand displayed two weak hypersensitive sites upstream to the strong site, while the top strand displayed one additional strong hypersensitive site at the 3' border of the footprint. The shorter top strand footprint was contained completely within the longer protected region of the bottom strand.

Enhancer Activity of the Ovalbumin Promoter —1100 Fragment—To test the functional activity of the -1100 fragment, this 220-bp fragment was inserted upstream of various promoters fused to the chloramphenicol acetyltransferase (CAT) gene. The plasmids used were pAloCAT2 (SV40 early promoter with no enhancer), pBLCAT2 (tk promoter), and pOVCATMA (ovalbumin promoter extending from +41 to -723). The -1100 fragment was inserted into these vectors in both orientations and as dimers (Fig. 2). The resulting recombinant plasmids were transfected into CVI cells. Fig. 3, A and B show the effect of the -1100 fragment on expression of the SV40 and tk promoters. The -1100 fragment enhanced SV40 promoter activity 5-10-fold. The increased activity was independent of orientation (pSV220(-)) and copy number (pSV440(+)). The -1100 fragment did not significantly increase tk promoter activity (Fig. 3B). The slight decrease in CAT activity seen with the -1100 fragment in the correct orientation (pTK220(+)) is not reproducible. Fig. 3C shows that the -1100 fragment increased ovulamin promoter activity about 3-fold; this effect was orientation-independent.

Steroid-independent Enhancer Activity of the Ovalbumin -1100 Fragment—Injection of chicks with steroids results in more than a 1000-fold increase in ovalbumin mRNA production (O'Malley et al., 1969, 1979; Palmeter, 1972). To determine the response of the -1100 fragment to steroids, the SV40 promoter constructions containing the -1100 fragment were cotransfected into CVI cells with plasmids expressing either the chicken progesterone receptor (Conneely et al., 1987) or the human estrogen receptor (Walter et al., 1985). The plasmids have higher CAT activity than pAloCAT2, pSV220(-), or pSV220(+) was detected in the presence or absence of the progesterone receptor and hormone. Likewise, transfection with the human estrogen receptor gene expression vector did not alter the levels of expression of these plasmids (data not shown). Neither the estrogen nor the progesterone receptors stimulated activity of the tk promoter constructions (data not shown). The activity of the ovalbumin promoter from +41 to -723 (pOVCATMA) is stimulated in CVI cells by cotransfection with expression vectors containing either the estrogen or estrogen receptor genes. The presence of the -1100 fragment increased overall expression over that of steroid-dependent stimulation of pOVCATMA (data not shown). Thus, the -1100 enhancer activity is steroid-independent for the SV40 and ovalbumin promoters in transfected CVI cells.

The DNase I Protected Sequence Is Responsible for Enhancer Activity—To test whether the enhancer activity of the -1100 fragment was due to the DNase I protected region and not due to some other sequences in the 220-bp fragment, an oligonucleotide containing the sequences footprinted by oviduct nuclear extract (-1095 to -1130) was synthesized and cloned into pAloCAT. The resulting plasmids, pSV36-1 and pSV36-2, are independent clones of the same construction. These plasmids have higher CAT activity than pAloCAT2 (Fig. 5). The oligonucleotide has also been cloned in the reverse orientation and had similar CAT activity (data not shown). This activity is comparable to that of the entire fragment, suggesting that the DNase I-protected sequence (-1100 oligonucleotide) is responsible for the enhancer effect.

The -1100 Region Binding Protein is Related to NF-1—The consensus recognition sequence for NF-1 is TGGN₆-GCCAA (Leegwater et al., 1980). The DNase I-protected region of the -1100 fragment contains one-half of this consensus sequence, GCCAA, in the lower strand (Fig. 1B). Since Jones et al. (1987) have demonstrated that NF-1 binding is primarily dependent upon the GCCAA half-site, we wanted to determine whether the oviduct factor binding to the -1100 fragment is related to NF-1. Band-shifting competition assays

D. Kettelberger, unpublished observation.
Fig. 1. DNase I footprinting of the ovalbumin −1100 fragment. A, the −1100 fragment (−953 to −1174) of the ovalbumin gene was 3′ end-labeled on the top strand and 5′ end-labeled on the bottom strand and then incubated in the presence of increasing volumes (microliter) of the DE175 fraction of oviduct nuclear extract (3 mg/ml) prior to DNase I digestion. Nucleotide positions were determined by sequencing lanes (not shown). Brackets indicate sequences protected from DNase I digestion, whereas arrows point to hypersensitive sites. B, brackets indicate the positions of the strongest footprints on both the upper and lower strands. Small arrows point to hypersensitive sites. The leftward pointing arrow indicates the NF-1 half-site in the lower strand. Nucleotide positions relative to the ovalbumin cap site are indicated below the sequence.

Fig. 2. Schematic of CAT reporter gene constructions. The −1100 fragment (−953 to −1174) of the ovalbumin (OV) gene (open arrows) and a 36-bp synthetic oligonucleotide containing the DNase I-protected region of the −1100 fragment (solid arrows) were inserted into promoter-CAT fusion vectors; position, orientation, and number are indicated by the arrows. Vectors used were pAαgCAT2 (enhancer-less SV40 promoter), pBLCAT2 (tk promoter), and pOVCATMA (ovalbumin promoter sequences from +41 to −732).

Fig. 3. Enhancer activity of the ovalbumin −1100 fragment. CV1 cells were transiently transfected with 10 µg of the indicated reporter gene construction (see Fig. 2). Autoradiograms of thin layer chromatograms indicate CAT activity as the conversion of non-acetylated [14C]chloramphenicol (lowest mobility) to acetylated forms (higher mobility). Mass of protein assayed: 25 µg (A and B) or 100 µg (C). Autoradiographic exposure: 18 h (A and B) or 4 days (C). Lane B represents CAT activity of mock-transfected cells.

were used for this purpose. The −1100 fragment was end-labeled and mixed with unlabeled oligonucleotides with the sequence for either the −1100 footprint region, the adenovirus 2 NF-1 binding site (DeVries et al., 1987), or the COUP site (Sagami et al., 1986) before addition of oviduct DE175 fraction. Fig. 6A shows that a 50-fold molar excess of the −1100 oligonucleotide competed for DE175 factor binding by over 50%, while the NF-1 oligonucleotide completely abolished factor binding. In contrast, the COUP oligonucleotide, which...
Steroid-independent enhancer activity of the ovalbumin -1100 fragment. CV1 cells were cotransfected with 10 μg of the indicated reporter gene construction (see Fig. 2) and 5 μg of progesterone receptor (Pr. Rc.) gene expression vector (+), or its vector control (−). Assay conditions were as described in Fig. 3, A and B.

The oligonucleotides for both NF-1 and the -1100 footprinting region were end-labeled and used as probes in band-shifting competition assays (Fig. 6B). The major shifted band for both oligonucleotides migrated to the same position in the gel. As expected, the unlabeled -1100 region oligonucleotide competed very well for factor binding to the -1100 probe. It competed less well for binding to the NF-1 oligonucleotide probe. The unlabeled NF-1 oligonucleotide completely abolished factor binding to the -1100 oligonucleotide probe and almost completely abolished binding to the NF-1 oligonucleotide probe. In control lanes, an oligonucleotide spanning from -215 to -255 of the ovalbumin 5′-flanking sequence (−220) did not compete for binding to either probe. These data suggest that the same protein in the oviduct DE175 fraction binds to both the -1100 footprinting sequence and the NF-1 binding site, but that the affinity of the factor to the adenovirus origin of replication, which contains a complete NF-1 consensus recognition sequence, is stronger.

DISCUSSION

A factor in oviduct nuclear extract binds to a region between -1094 to -1125 of the ovalbumin promoter. This -1100 region enhances transcriptional activity 5–10-fold in a hormone-independent manner when placed upstream of the SV40 or the ovalbumin promoter in transiently transfected CV1 cells. Finally, band-shifting competition assays with oligonucleotides for the adenovirus 2 NF-1 site and the -1100 footprint region show that this factor from oviduct nuclei is, or is related to, nuclear factor-1.

The -1100 fragment acts as an enhancer when placed immediately adjacent to the SV40 promoter (pSV220). It also enhances transcriptional activity when placed over 700 bp upstream of ovalbumin proximal promoter (pOV220MA). We have not shown here that the enhancer works in the normal position of 1100 bp upstream of the ovalbumin gene. However, in the ovalbumin promoter construction (pOV220MA), the enhancer is less than 200 bp downstream of its natural site, suggesting that it would work in its normal position.

The -1100 binding sequence contains only the GCCAA half of the NF-1 consensus recognition sequence (TGGN_{4-7} GCAA; Leegwater et al., 1986). However, Jones et al. (1987) have noted that the GCCAA part of the sequence controls recognition by NF-1/CTF, while the 2-fold symmetry created by the presence of the TGG sequence greatly increases the affinity of the protein. Rosenfeld and Kelly (1986) have shown
that mutating the adenovirus origin of replication NF-1 binding sequence to perfect symmetry increases NF-1 affinity 4-fold. This is consistent with the data presented herein, since an oligonucleotide to the adenovirus 2 NF-1 site, with near-perfect symmetry, competes for factor binding to the −1100 region of the promoter with a fivefold affinity than the oligonucleotide for the −1100 region itself.

NF-1 was first identified as an essential factor for adenovirus replication (Nagata et al., 1983). NF-1 sites have since been described in the 5’-flanking region of several other genes, including the Herpes simplex virus tk promoter, the MMTV long terminal repeat, the chicken lysozyme gene, the human c-myc gene, and the BK virus enhancer (Borgmeyer et al., 1984; Nowock et al., 1985). The common association of these sites with regions of DNase I hypersensitivity suggests that NF-1 proteins are important in transcriptional activation (Borgmeyer et al., 1984). Miksicek et al. (1987) have shown that point mutations in the NF-1 binding site of the MMTV long terminal repeat resulted in decreased glucocorticoid-induced expression of the MMTV promoter in vivo. Deletion of the NF-1 sequence in the mouse α2(I) collagen promoter also reduces activity 4-fold in transient transfection assays (Oikarinen et al., 1987; Schmidt et al., 1986). Our work demonstrates that placing the ovalbumin NF-1 site upstream of the ovalbumin or SV40 promoter increases the activity of both 5-10-fold in transient transfection assays. However, no enhancement was seen when the −1100 sequence was placed upstream of the tk promoter. The lack of increased tk promoter activity may be due to an endogenous NF-1/CTF site in this promoter (Jones et al., 1987).

The oviduct nuclear protein which binds to the −1100 region of the ovalbumin gene may be a member of a family of proteins. The existence of such a family is supported by the observation that the NF-1/CTF factor purified from HeLa cells (Jones et al., 1985), as well as the NF-1 protein purified by Rosenfeld and Kelly (1986), are families of polypeptides ranging between 52 and 66 kDa. Furthermore, the NF-1/CTF factor binds to the tk and α2-globin CCAAT boxes, but does not bind to the CCAAT box of certain other genes including the α2(I) collagen promoter (Oikarinen et al., 1987). Similarly, the TGGCA/NF-1 protein from the chicken liver binds to the MMTV long terminal repeat NF-1 site, but does not bind to the tk CCAAT box (Miksicek et al., 1987). Accumulating evidence indicates that the CCAAT box is recognized by a multiplicity of factors (Dorn et al., 1987), and NF-1 may be a member of that family.

We have identified a hormone-independent ovalbumin enhancer. However, the large induction of ovalbumin gene expression in hormonally stimulated chickens cannot be fully explained by this enhancer or any other single cis- or trans-acting factor yet identified. A combination of factors appears to interact with each other to effect the response. Several lines of evidence support this hypothesis. First, multiple factors from oviduct nuclei bind specifically to the ovalbumin 5’-flanking sequence. The function of most of these factors is currently unclear, although some may be involved in steroid receptor regulation and others may be involved in repression of gene expression. Secondly, the effects of many enhancers interact with those of silencers. It appears that a sequence of the ovalbumin promoter acts as a silencer which is derepressed by steroid-regulated elements (Gaub et al., 1987). Similarly, silencer elements of the chicken lysozyme gene can be compensated by enhancer elements (Baniahmad et al., 1987). Finally, when the −1100 enhancer is placed upstream of steroid-responsive regions of the ovalbumin gene (between +41 and −732), the overall level of gene transcription is increased above the level achieved by the steroid-regulated region alone in the presence of hormones. These data indicate that a combination of general enhancers, such as the −1100 region, may interact with steroid-regulated enhancers to achieve the observed large increase in the overall level of gene activation.

We suggest the following hypothesis for overall regulation of ovalbumin gene expression. Silencer or repressor elements permit little to no basal expression of the ovalbumin gene. Steroid-regulated enhancers override this repression in the presence of steroids. Finally, steroid-independent enhancers, such as the NF-1 enhancer defined in this paper, synergistically increase steroid-induced transcriptional activity. This combination of cis-acting elements leads to a gene which is highly controlled in the absence of hormone, but which can be induced by steroids to a high level of expression whereby ovalbumin mRNA represents almost one-half of the total mRNA pool. This hypothesis is currently under investigation in our laboratory.

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Ovalbumin Enhancer Binds NF-1-like Factor