YY1 binds to regulatory element of chicken lysozyme and ovalbumin promoters

Mahboob Morshed · Munetoshi Ando · Junko Yamamoto ·
Akitsu Hotta · Hidenori Kaneoka · Jun Kojima ·
Ken-ichi Nishijima · Masamichi Kamihira · Shinji Iijima

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Abstract Chicken lysozyme is highly expressed in the oviduct. The 5' regulatory region of this gene contains a negative element that represses transcription. To assess the molecular basis underlying the regulation of lysozyme gene expression, we investigated the binding protein to this region. Sequence motif analysis suggested the existence of putative YY1 binding sites in this regulatory region. Electrophoretic mobility shift assay showed the specific binding of YY1 to the negative element. In addition, chromatin immunoprecipitation assay indicated that YY1 specifically bound to the negative element in oviduct cells but not in erythrocytes. It was suggested by electrophoretic mobility shift assay and chromatin immunoprecipitation assay that YY1 also bound to the negative regulatory region in the promoter of the ovalbumin gene which also shows oviduct-specific expression. Western blot analysis showed

that YY1 was expressed in relatively high levels in the oviduct and nucleus fractionation experiments showed that YY1 was localized both in chromosomal and nuclear matrix fractions. These results suggest that there are some specific roles in the negative regulatory regions of these genes in relation to the multifunctional transcription factor YY1.

Keywords Lysozyme · Ovalbumin · Oviduct · Promoter · YY1

Abbreviations

ChIP	chromatin immunoprecipitation
DHS	DNase I-hypersensitive site
EMSA	electrophoretic mobility shift assay
NE	negative element
NRE	negative regulatory element
SDRE	steroid dependent regulatory element

M. Morshed · M. Ando · J. Yamamoto ·
A. Hotta · H. Kaneoka · J. Kojima ·
K. Nishijima (✉) · S. Iijima
Department of Biotechnology, Graduate School
of Engineering, Nagoya University, Furo-cho,
Chikusa-ku, Nagoya 464-8603, Japan
e-mail: nishijima@proc.nubio.nagoya-u.ac.jp

M. Kamihira
Department of Chemical Engineering, Faculty of
Engineering, Kyushu University, Motooka, Nishi-ku,
Fukuoka 819-0395, Japan

Introduction

Chicken egg white contains several proteins in vast amounts which are produced in the oviduct tissues. Lysozyme and ovalbumin are the major proteins in egg white and these genes have been used as excellent models to investigate the control mechanisms of hormonally and developmentally regulated gene expression (Muramatsu and

Sanders 1995; Short et al. 1996; Bonifer et al. 1997; Dillner and Sanders 2000).

The lysozyme locus in the chicken chromosome is about 24 kb in length and contains both positive and negative regulatory elements which act coordinately (Short et al. 1996; Bonifer et al. 1997). Lysozyme is expressed both in tubular gland cells in the oviduct and mature macrophages, and the expression is induced by steroid hormones including estrogen and progesterone in the oviduct while the expression in macrophages is developmentally regulated and independent of the steroid hormones (Short et al. 1996).

In oviduct cells, five DNase I-hypersensitive sites (DHSs) have been detected centered at – 0.1 kb, – 1.9 kb, – 2.4 kb, – 6.1 kb and – 7.9 kb in the 5' flanking region of the chicken lysozyme gene. DHS at – 2.4 kb is present in oviduct cells and also in other cells that do not express the lysozyme gene such as liver, kidney and brain. Therefore, it has been proposed that the role of this DHS is not to activate the lysozyme gene but related to suppression (Fritton et al. 1984). However, the suppressor proteins which bind to this DHS in oviduct have not been elucidated. In macrophage lineage, a different array of DHSs has been reported: In mature macrophages, DHSs at – 6.1 kb, – 3.9 kb, – 2.7 kb, – 0.7 kb and – 0.1 kb are observed which are essential for developmentally regulated expression of this gene. The DHS at – 2.4 kb was detected in myeloblasts that do not express lysozyme but the site disappears at a subsequent stage of differentiation and a new DHS at – 2.7 kb is formed accompanying with the expression of the lysozyme gene (Fritton et al. 1987). Thus the DHS at – 2.4 kb is called the negative element (NE) and it represses the gene expression in lysozyme non-producing cells (Bonifer et al. 1997).

Within 8.7 kb of the DNA region between ovalbumin and the upstream neighboring gene called Y gene, there are four DHSs located at 0.15 kb, 0.8 kb, 3.2 kb and 6.0 kb upstream from the transcription start site of the ovalbumin gene (Kaye et al. 1986). Two major regulatory elements reside in the chicken ovalbumin gene. One is a steroid-dependent regulatory element (SDRE), the other is a negative regulatory element (NRE) (Sanders and McKnight 1988).

DHS II corresponds to SDRE (spanning from – 892 to – 780) which is required for responsiveness to estrogen and glucocorticoid. DHS I corresponds to NRE (spanning from – 88 to – 308) and appears to have a dual role of repressing and activating transcription (Sanders and McKnight 1988; Ehlen Haecker et al. 1995). Fine mapping of this region suggested that NRE consisted of at least four different elements (Ehlen Haecker et al. 1995). Recent studies revealed that IRF-4 and δ EF1 are putative DNA binding proteins within NRE (Dillner and Sanders 2000, 2002).

YY1 is a multifunctional transcription factor that either represses or enhances the transcription of a variety of cellular and viral genes and initiates DNA replication of the adeno-associated virus (Shi et al. 1997; Thomas and Seto 1999). Since YY1 is expressed ubiquitously in mammals and participates in the regulation of many developmentally regulated genes, we analyzed the expression and localization of YY1 in the oviduct and the binding of YY1 to the lysozyme and ovalbumin promoters.

Materials and methods

Reagents

Anti-YY1 (sc-281 X), anti-NF1 (sc-870) and control rabbit IgG (sc-2027) antibodies were purchased from Santa Cruz Biotechnology, CA, USA. Anti-acetyl-histone H3 antibody (#06-599) was purchased from Upstate cell signaling solutions, VA, USA.

Cloning of chicken YY1 cDNA

For cloning the C-terminal region of YY1, cDNAs which were obtained from chicken embryonic fibroblasts were amplified by KOD-Plus (Toyobo, Osaka, Japan) with the primers 5'-taaccatggcacaccaccaggaggtga-3' and 5'-ccgctgcagaagaatcgctctttttgatgcaa-3' (italic letters indicate *Pst*I and *Nco*I sites that were used for cloning) and the amplified DNA was cloned into pETBlue (Novagen, Darmstadt, Germany). For cloning of GC rich N-terminal region, genomic DNA was amplified by LA taq (Takara, Shiga, Japan) with

the primers 5'-gagcagactctgtccccctg-3' and 5'-taccgacagggccctatgtt-3'. The PCR fragment was cloned into pT7Blue-2 T-vector (Novagen). Both constructs were verified by DNA sequencing using ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA). To obtain whole YY1, the N-terminal region was amplified with the primers 5'-cctggatccaccatggcctcgga-3' and 5'-ctcAcgCgtctgcaccaggatca-3' (italic and capital letters indicate *Bam*HI site used for cloning and mutated nucleotides to introduce *Mlu*I site, respectively), and the C-terminal region was amplified with the primers 5'-gcagacGcgTgaggaggtgtg-3' and 5'-ggtgtcgactcaagaactttcctcaac-3' (capital and italic letters indicate mutated nucleotides to introduce *Mlu*I site and *Sal*I site used for cloning, respectively). Finally N-terminal and C-terminal regions were ligated at *Mlu*I site and the whole chicken YY1 cDNA was then ligated into *Bam*HI/*Xho*I sites of pcDNA4 (Invitrogen, CA, USA). The resultant plasmid, which expresses chicken YY1 under the control of CMV promoter, was designated as pcDNA4-cYY1. Due to this cloning strategy, silent mutations were generated at Arg 116 and Glu 117.

Western blotting

Tissues obtained from laying hens were sonicated in the presence of protease inhibitors (aprotinin 2 µg/ml, pepstatin A 1 µg/ml and phenyl methylsulfonyl fluoride 100 µg/ml) to obtain the whole cell lysate. The protein concentrations were determined using bicinchoninic acid (Sigma Chemical, MO, USA) with bovine serum albumin as a standard. An equal amount of protein was loaded on each lane of a 7.5% polyacrylamide gel and transferred to PVDF membrane followed by the detection with anti-YY1 antibody as reported previously (Nishijima et al. 2005).

Nuclear extract preparation

293FT cells were transfected with pcDNA4cYY1 or control pcDNA4 with LipofectaminTM 2000 (Invitrogen) following the supplier's protocol. After 48 h of culture, the cell pellet was suspended with 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and

0.6% NP40 and the suspension was incubated for 15 min on ice and centrifuged at $20,000 \times g$ for 30 s at 4°C. Pellet containing nuclei was suspended with 20 mM HEPES-KOH, pH 7.9, 400 mM NaCl, 1 mM EDTA and 1 mM EGTA and the suspension was incubated for 15 min at 4°C. Supernatant was recovered as nuclear extract after centrifugation at $20,000 \times g$ for 15 min at 4°C. Nuclear extract was diluted twice with the same buffer without NaCl to reduce the salt concentration to 0.2 M. This sample was used for electrophoretic mobility shift assay (EMSA).

Nuclei were prepared from the oviduct of laying hens by homogenizing the tissue with a glass homogenizer followed by sequential centrifugations in TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl and 5 mM MgCl₂) with different concentrations of sucrose as reported previously (Spelsberg et al. 1974). In brief, 25 g of oviduct tissue was minced and homogenized in TKM buffer containing 0.5 M sucrose and then the homogenized tissue was filtrated and centrifuged at $10,000 \times g$ for 5 min. The pellet was again homogenized in TKM buffer containing 1.7 M sucrose and centrifuged at $20,000 \times g$ for 10 min (twice). The recovered pellet was homogenized in TKM buffer containing 0.5 M sucrose and 0.2% Triton X-100, the sample was filtrated and then centrifuged for 10 min at $10,000 \times g$. The pellet (nuclei) was suspended in 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂ and 25% glycerol. For the preparation of nuclear extract, nuclei were washed with phosphate buffered saline (PBS), then suspended in PBS containing 0.4 M NaCl and incubated for 15 min at room temperature. Nuclear extract was recovered as the supernatant after centrifugation at $10,000 \times g$ for 10 min, followed by extensive dialysis against 20 mM HEPES-KOH, pH 7.9, 50 mM KCl, 0.2 mM EDTA and 20% glycerol. All buffers contained protease inhibitors (aprotinin 2 µg/ml, pepstatin A 1 µg/ml and phenyl methylsulfonyl fluoride 100 µg/ml).

Fractionation of the nuclei

Nuclei were fractionated as reported previously (Pasqualini et al. 2001) with some modifications. Briefly, after the isolation of the nuclei from the

oviduct tissues, the nuclei were washed once with PBS and then suspended in five volumes of ice-cold CSK buffer (10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8, 300 mM sucrose, 3 mM MgCl₂, 100 mM NaCl, 1 mM EGTA, 1 mM phenyl methyl-sulfonyl fluoride, 20 µg/ml aprotinin and 10 µg/ml pepstatin A). After centrifugation at 350 × *g* for 5 min at 4°C, the nuclei were resuspended in CSK buffer containing 1 M sucrose and centrifuged at 1,200 × *g* for 10 min at 4°C. From this pellet, soluble proteins (nucleoplasm) were extracted with the CSK buffer containing 0.5% Triton X-100 for 5 min at 4°C followed by centrifugation at 5,000 × *g* for 10 min at 4°C. The pellet was then digested with DNase I (700 U/ml) in CSK buffer containing 50 mM NaCl for 60 min at 4°C. The chromatin-associated proteins were eluted by slowly adding ammonium sulfate in the solution to a final concentration of 0.25 M. The nuclear matrix was pelleted by centrifugation at 5,000 × *g* for 5 min at 4°C and the chromatin fraction was isolated as a supernatant. The nuclear matrix was solubilized in 8 M urea at pH 8. Approximately 33%, 51% and 16% of the nuclear proteins were recovered as nucleoplasm, chromatin and nuclear matrix fractions, respectively. Equal proportions of each fraction were subjected for Western blotting analysis.

EMSA

Following oligonucleotides were used as probes: From –2539 to –2512 of lysozyme gene, 5'-gatc ttcatttcttccatgttggtgaca-3' and 5'-gtgtcaccaacatgga agaataagaat-3'; from –153 to –125 of ovalbumin gene, 5'-gctccattcaatccaaatggacattga-3' and 5'-gtcaataggtccatttggattgaatggag-3'; from –146 to –120 of ovalbumin gene, 5'-gcaatccaaatg gacctattgaaacta-3' and 5'- gtagtttcaataggtccatttgg attg-3'; from –165 to –139 of ovalbumin gene, 5'-gctaataatttgcctccattcaatccaa-3' and 5'-gttgattga atggagagcaatattag-3' in which italic letters indicate added nucleotides. The oligonucleotides were annealed and end-labeled with ³²P-γ-ATP using T4 polynucleotide kinase (Takara). Binding reactions were carried out in a final volume of 15 µl containing ³²P-labeled DNA probe (approximately 10,000 cpm) and 300 ng of poly (dI/dC), 2 mM MgCl₂, 100 ng BSA, 20% glycerol

and nuclear extract (either 293FT nuclear extract containing 2 µg of protein or oviduct nuclear extract containing 40 µg protein). The mixture was incubated on ice for 40 min. Protein-DNA complexes were resolved by electrophoresis on a 6% polyacrylamide gel at 100 V for 5 h in 40 mM Tris, 20 mM acetic acid and 1 mM EDTA. For competition experiments, a 1000-fold molar excess of unlabeled specific or control oligonucleotides were added to the reaction mixture prior to the addition of the nuclear extract. For supershift assays, nuclear extract was incubated with 5 µg of anti-YY1 antibody on ice for 30 min prior to the addition of the labeled probe.

Chromatin immunoprecipitation (ChIP) assay

Cells were prepared from the oviduct of estrogen-induced immature chickens as reported previously (Sanders and Mcknight 1985). ChIP was performed for the oviduct cells and erythrocytes from laying hen as described previously (Inayoshi et al. 2005) using anti-YY1 and control rabbit IgG antibodies. The following primers were used for amplification: For NE of lysozyme, 5'-caaagcagga gttagcgg-3' and 5'-ctgggtcaataagtaactaagc-3' for direct and reverse primers, respectively; for NRE of ovalbumin, 5'-aagctcaatggaacatgagca-3' and 5'-atcatttaatgggattgggttaga-3' for direct and reverse primers, respectively; for β-globin, 5'-aggtcaatgtg ccgaatgt-3' and 5'-ggtgagcactttcttccgt-3' for direct and reverse primers, respectively.

Results

Expression of YY1 in the chicken oviduct

YY1 is a highly conserved protein and contains four zinc fingers located at the C-terminal region which are responsible for sequence-specific DNA binding. The C-terminal domain is identical for humans and chickens (Sui et al. 2004). Thus, the anti-YY1 antibody toward the C-terminal region of human YY1 reacts with the chicken YY1 which enabled us to assess the expression levels of YY1 in different chicken tissues by Western blotting. As shown in Fig. 1A, YY1 was detected in various tissues when the whole cell extract was analyzed.

Among the tissues, the oviduct cells expressed high levels of YY1 and two bands showing different mobility were detected. In oviduct nuclear extracts, however, a high molecular weight band was observed which showed the same mobility as cloned YY1 (Fig. 1B). Since YY1 was shown to be mainly localized in the nuclear matrix in several cell lines (Guo et al. 1995; Bagchi et al. 1998), the localization of YY1 in the nucleus of the oviduct cells was then examined. Isolated nuclei were further fractionated into nucleoplasm, chromatin and nuclear matrix fractions. As shown in Fig. 1C, more than half of YY1 was detected in the nuclear matrix fraction while a lesser amount was associated with the chromatin. Acetylated histone H3 and NF1 were also analyzed as marker proteins for the chromatin and nucleoplasm fractions, respectively. It was confirmed that acetylated

histone H3 was detected only in the chromatin fraction while NF1 was detected only in the nucleoplasm fraction.

YY1 binds to NE of the lysozyme promoter

Using computer analysis of NE region of the 5' flanking region of the lysozyme gene with the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>), three putative YY1 binding sites were identified with the highest TFSEARCH scores from -2540 to -2510 (Fig. 2B). Two sites were found to be on the coding strand and the third one on the complementary strand. The binding of YY1 to NE was examined by EMSA using a nuclear extract from 293FT cells that had been transfected with an expression vector of chicken YY1. The expression

Fig. 1 Chicken YY1 is expressed ubiquitously in chicken tissues and is localized in nuclear matrix and chromatin fractions in oviduct. **(A)** Whole cell extract (20 μ g) was subjected for Western blotting with anti-YY1 antibody. Nuclear extract of 293FT cells (0.5 μ g) was also loaded for comparison. **(B)** Nuclear extract from 293FT cells transfected with chicken YY1 (1 ng, Rec YY1), whole (12 μ g) and nuclear (0.5 μ g) extracts from oviduct were subjected for Western blotting. **(C)** Nuclear fractions were subjected for Western blotting by anti-YY1 (top), anti-acetyl-histone H3 (Ac-H3, middle) and anti-NF1 (bottom) antibodies

Fig. 2 YY1 binds to NE in the lysozyme promoter. **(A)** Schematic representation of DHSs within 3 kb upstream from the transcription start site of the chicken lysozyme gene. NE (–2540 to –2231) is indicated as hatched box. **(B)** Putative YY1 binding sites within NE. The consensus sequences for YY1 binding are shown at left (Hyde-DeRuyscher et al. 1995). Nucleotide sequence from –2540 to –2501 in lysozyme upstream region is indicated with the arrows corresponding to putative YY1 binding sites. Oligonucleotide used as the probe in EMSA is boxed. **(C)** Confirmation of YY1 expression in 293FT cells transfected with the expression vector for chicken YY1 (pcDNA4cYY1). Endogenous YY1 was weakly detected

in the cells that were transfected with control vector (ctrl). **(D)** YY1 binds to NE. EMSA was performed with nuclear extract from 293FT cells transfected with either pcDNA4cYY1 (YY1) or control vector (ctrl). **(E)** Confirmation of the specific binding of YY1 to NE. Extract from 293FT cells that had been transfected with pcDNA4cYY1 were used. Oligonucleotide that is identical to probe (NE) or oligonucleotide that does not contain putative YY1 binding sequence (ctrl) was used as competitor. Arrows and arrowhead indicate the specific shifted bands and the supershifted band in the presence of anti-YY1 antibody, respectively

Fig. 3 YY1 strongly binds to the site 3 in NE of the lysozyme promoter.

(A) Schematic representation of putative YY1 binding sites 1, 2 and 3 in NE. (B) Sequences and locations of the probe and the competitors for EMSA. Mutated nucleotides are underlined. Competitor 'h' is the consensus sequence for YY1 binding. (C) EMSA was performed with nuclear extract from 293FT cells transfected with pcDNA4cYY1. Arrow and arrowhead indicate the specific shifted band and the supershifted band in the presence of anti-YY1 antibody, respectively

of YY1 was confirmed by Western blotting (Fig. 2C). As shown in Fig. 2D, a shifted band emerged with a nuclear extract from 293FT cells that overexpressed chicken YY1. The band was specifically eliminated by the addition of identical oligonucleotide but not of an oligonucleotide without binding sequence of YY1 (Fig. 2E). Furthermore, a supershifted band appeared by the addition of anti-YY1 antibody. The results demonstrate that YY1 specifically binds to NE of the lysozyme promoter.

To identify more precisely which sequences are bound by YY1, we designed six competitors. In Fig. 3C, competitor 'b' which corresponds to the sequence from -2530 to -2515 and contains sites 2 and 3 significantly reduced the intensity of the shifted band (lane 4). Competitor 'd' which has only site 3 by mutations in the site 2 reduced the intensity of the band (lane 6). On the other hand, competitor 'c' which has only site 2 by mutations in the site 3 showed slight or no effects on YY1 binding (lane 5). Furthermore, the shif-

ted band did not disappear by competitor 'e' which has mutations in both sites 2 and 3 (lane 7). On the other hand, competitor 'f' which contains only site 1 weakly decreased the intensity of the shifted band and competitor 'g' in which site 1 was mutated did not affect on the shifted band (lanes 8 and 9, respectively). For a positive control, an oligonucleotide with consensus sequence for YY1 binding was used as a competitor 'h' (lane 10), which removed the shifted band completely. Together, these results suggested that the primary binding site for YY1 is the site 3. The site 1 appeared to have a weaker binding capacity to YY1 and the binding activity of the site 2 may be much less. We then performed similar EMSA with the nuclear extract from oviduct tissues. As shown

in Fig. 4A, shifted band(s) appeared by the addition of the nuclear extract. The shifted band(s) disappeared by the addition of the identical oligonucleotide as competitor and a supershifted band was observed with anti-YY1 antibody, suggesting that YY1 in the oviduct cells specifically binds to NE of the lysozyme promoter.

To confirm the binding of chicken YY1 to NE of the lysozyme promoter *in vivo*, ChIP assay was performed. As shown in Fig. 4B, anti-YY1 antibody precipitated DNA including NE of the lysozyme promoter but not the β -globin gene. On the other hand, NE was not precipitated by anti-YY1 antibody in erythrocytes that do not express lysozyme. This result suggests that YY1 binds to NE of the lysozyme promoter in oviduct cells that express lysozyme.

Fig. 4 YY1 binds to NE in the oviduct. **(A)** Oviduct nuclear extract containing YY1 binds to NE. EMSA was performed as was in Fig. 2E with nuclear extract from oviduct. Arrows and arrowhead indicate the specific shifted bands and a supershifted band in the presence of anti-YY1 antibody, respectively. **(B)** ChIP analysis indicated that YY1 binds to NE of the lysozyme *in vivo*. Oviduct cells from estrogen-induced immature chickens were subjected for ChIP. NE of lysozyme but not β -globin was precipitated with anti-YY1 antibody (upper column). In erythrocytes, which do not express lysozyme, anti-YY1 antibody did not precipitate NE (lower column). Input (10%) was used as a positive control

YY1 binds to NRE of the ovalbumin promoter

Ovalbumin is a protein that is specifically and strongly expressed in the oviduct. There are three DHSs within 4 kb upstream from the transcription start site (Fig. 5A top) that do not appear in other tissues (Kaye et al. 1986). DHS I contains a NRE which consists of four independently acting negative elements (–280 to –252, –237 to –228, –175 to –132 and –132 to –87) (Ehlen Haecker et al. 1995). By TFSEARCH analysis, we identified two putative YY1 binding sites with the highest scores within NRE. One site was found on the coding strand overlapping with δ EF1 binding site and the other site on the complementary strand (Fig. 5A bottom). Therefore, we assessed whether YY1 binds to this region of the ovalbumin promoter by EMSA. A specific shifted band was observed by the addition of nuclear extract from 293FT cells expressing chicken YY1 (Fig. 5B). The shifted band disappeared by the addition of identical oligonucleotide. Furthermore, a supershifted band emerged by the addition of anti-YY1 antibody, suggesting that YY1 binds to NRE in the ovalbumin promoter.

To confirm the binding of YY1 to ovalbumin NRE *in vivo*, we performed a ChIP assay. As shown in Fig. 5C, anti-YY1 antibody specifically precipitated the DNA fragments containing NRE in oviduct cells although the intensity was lower than that in lysozyme NE.

Fig. 6 YY1 strongly binds to the site 2 in NRE of the ovalbumin promoter. **(A)** Schematic representation of putative YY1 binding sites 1 and 2 in NRE. **(B)** Sequences and locations of the probes and the competitors for EMSA. Lower cases indicate the added nucleotide. Mutated nucleotides are underlined. Competitor ‘e’ which contains the consensus sequence for YY1 binding was used as positive control. **(C)** EMSA was performed using an

oligonucleotide corresponding to the sequence from – 165 to – 139 as probe with the nuclear extract from 293FT cells that had been transfected with pcDNA4cYY1. **(D)** EMSA was performed using an oligonucleotide corresponding to the sequence from – 146 to – 120 as probe. Arrows and arrowhead indicate the specific shifted bands and the supershifted band by anti-YY1 antibody, respectively

‘c’ where competitor ‘a’ contains only site 2 and competitor ‘c’ contains both sites 1 and 2 (lanes 3 and 5, respectively). The intensity of shifted band did not change by competitor ‘b’ that contains mutated nucleotides in the site 2 (lane 4). The shifted band did not disappear by the addition of competitor ‘d’ that has only site 1, confirming again that YY1 did not bind this site (lane 6). No shifted band was observed when competitor ‘e’ was used as a positive control which contains consensus sequence for YY1 binding (lane 7). This result suggests that the primary binding site for YY1 within NRE is the site 2. Together, these results suggest that YY1 specifically binds to ovalbumin NRE in the oviduct.

Discussion

In this study, using EMSA assay and ChIP analysis, we demonstrated that YY1 binds to chicken lysozyme DHS centered at -2.4 kb in the oviduct. This DHS was detected in oviduct cells as well as lysozyme non-expressing cells such as liver and kidney cells but not in erythrocytes (Fritton et al. 1984). Since the appearance of the DHS does not show specificity for lysozyme-expressing cells, its function was thought to be repression (Fritton et al. 1984). In the present study, we demonstrated that YY1 bound to NE, suggesting that the binding of YY1 represses gene expression. However, we cannot rule out the possibility that the binding of the other transcription factors represses the gene expression. So far, the function of the lysozyme NE was explained in developing macrophages. During differentiation, macrophages lose the DHS at -2.4 kb and start to express lysozyme (Fritton et al. 1984; Steiner et al. 1987). The multifunctional protein CTCF and the thyroid hormone receptor or the retinoic acid receptor are reported as putative binding proteins (Banahmad et al. 1990; Burcin et al. 1997). On the other hand, the DHS at -2.4 kb is present both in mature and immature oviduct cells (Fritton et al. 1984). In this regard, it is interesting to assess whether YY1 binds to the lysozyme NE region in immature macrophages.

YY1 is indispensable for cell cycle progression (Sui et al. 2004) and ubiquitous expression is

typical for the mammalian species (Shi et al. 1997; Thomas and Seto 1999). YY1 is known to be a multifunctional protein which regulates a variety of genes in both a positive and a negative manner depending on various factors such as cell types and promoters. The relationship between YY1 and chromatin modifying factors like histone deacetylase, histone acetyltransferase as well as various transcription factors (Shi et al. 1997; Thomas and Seto 1999) suggests that the regulatory mechanisms of YY1 may be very complicated. Thus, we cannot clarify the function of YY1 with the ovalbumin promoter although the binding sequence resides in NRE. In fact, NRE may contain binding sites for both negative and positive regulatory proteins. Sanders et al. postulated that δ EF1 binds to NRE and SDRE and activates ovalbumin expression in a hormone-dependent manner (Chamberlain and Sanders 1999; Dillner and Sanders 2002). The binding site for YY1 is located just next to the δ EF1 binding site in NRE. Further analysis is necessary to understand the function of YY1 in relation to δ EF1 for the control of the ovalbumin gene expression.

The nuclear matrix is postulated to be the scaffold that takes part in the regulation of various aspects of the gene expression and repression and specific DNA regions called the nuclear matrix attachment regions bind to the nuclear matrix (Bode et al. 2003). In the lysozyme locus, the nuclear matrix attachment regions lying upstream and downstream of the transcribed region have been well characterized (Short et al. 1996; Bonifer et al. 1997). In addition, several regions of the lysozyme and ovalbumin genes including their promoter regions bind to the nuclear matrix when they are transcribed (Ciejek et al. 1983; Phi-Van and Strätling 1988). It is therefore noteworthy that more than half of YY1 existed in the nuclear matrix in the oviduct in our analysis since both the nuclear matrix and the nonmatrix fractions of YY1 have binding ability to DNA (Guo et al. 1995; Bagchi et al. 1998). It is possible that YY1 has a certain role in binding specific genes to the nuclear matrix. To clarify the function of YY1, it is necessary to examine whether YY1 controls the binding of these loci to the nuclear matrix.

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References

- Bagchi M, Ansari SA, Lindenmuth DM, van Wijnen AJ, Lian J, Stein JL, Stein GS (1998) Nuclear matrix associated DNA-binding proteins of ocular lens epithelial cells. *Mol Biol Rep* 25:13–19
- Baniahmad A, Steiner C, Köhne AC, Renkawitz R (1990) Modular structure of a chicken lysozyme silencer: Involvement of an unusual thyroid hormone receptor binding site. *Cell* 61:505–514
- Bode J, Goetze S, Heng H, Krawetz SA, Benham C (2003) From DNA structure to gene expression: mediators of nuclear compartmentalization and dynamics. *Chromosome Res* 11:435–445
- Bonifer C, Jäggle U, Huber MC (1997) The chicken lysozyme locus as a paradigm for the complex developmental regulation of eukaryotic gene loci. *J Biol Chem* 272:26075–26078
- Burcin M, Arnold R, Lutz M, Kaiser B, Runge D, Lottspeich F, Filippova GN, Lobanenko VV, Renkawitz R (1997) Negative protein 1, which is required for function of the chicken lysozyme gene silencer in conjunction with hormone receptors, is identical to the multivalent zinc finger repressor CTCF. *Mol Cell Biol* 17:1281–1288
- Chamberlain EM, Sanders MM (1999) Identification of the novel players δ EF1 in estrogen transcriptional cascades. *Mol Cell Biol* 19:3600–3606
- Ciejek EM, Tsai M-J, O'Malley W (1983) Actively transcribed genes are associated with nuclear matrix. *Nature* 306:607–609
- Dillner NB, Sanders MM (2000) Hormone response units: the ovalbumin model. In: Pandalai SG (eds) Recent research developments in molecular & cellular biology, vol. 1, Kelara, India, pp 93–108
- Dillner NB, Sanders MM (2002) The zinc finger/homeodomain protein δ EF1 mediates estrogen-specific induction of the ovalbumin gene. *Mol Cell Endocrinol* 192:85–91
- Ehlen Haecker SA, Muramatsu T, Sensenbaugh KR, Sanders MM (1995) Repression of the ovalbumin gene involves multiple negative elements including a ubiquitous transcriptional silencer. *Mol Endocrinol* 9:1113–1126
- Fritton HP, Igo-Kemenes T, Nowock J, Strech-Jurk U, Theisen M, Sippel AE (1984) Alternative sets of DNase I-hypersensitive sites characterize the various functional states of the chicken lysozyme gene. *Nature* 311:163–165
- Fritton HP, Igo-Kemenes T, Nowock J, Strech-Jurk U, Theisen M, Sippel AE (1987) DNase I-hypersensitive sites in the chromatin structure of the lysozyme gene in steroid hormone target and non-target tissues. *Biol Chem Hoppe-Seyler* 368:111–119
- Guo B, Odgren PR, van Wijnen AJ, Last TJ, Nickerson J, Penman S, Lian JB, Stein JL, Stein GS (1995) The nuclear matrix protein NMP-1 is the transcription factor YY1. *Proc Natl Acad Sci USA* 92:10526–10530
- Hyde-DeRuyscher RP, Jebbubs E, Shenk T (1995) DNA binding sites for the transcriptional activator/repressor YY1. *Nucl Acids Res* 23:4457–4465
- Inayoshi Y, Kaneoka H, Machida Y, Terajima M, Dohda T, Miyake K, Iijima S (2005) Repression of GR-mediated expression of the tryptophan oxygenase gene by the SWI/SNF complex during liver development. *J Biochem* 138:457–465
- Kaye JS, Pratt-Kaye S, Bellard M, Dretzen G, Bellard F, Chambon P (1986) Steroid hormone dependence of four DNase I-hypersensitive regions located within the 7000-bp 5'-flanking segment of the ovalbumin gene. *EMBO J* 5:277–285
- Muramatsu T, Sanders MM (1995) Regulation of ovalbumin gene expression. *Poult Avian Biol Rev* 6:107–123
- Nishijima K, Ando M, Sano S, Hayashi-Ozawa A, Kinoshita Y, Iijima S (2005) Costimulation of T cell proliferation by anti-L-selectin antibody is associated with the reduction of a cdk inhibitor p27. *Immunology* 116:347–353
- Pasqualini C, Guivarc'h D, Barnier J-V, Guibert B, Vincent J-D, Vernier P (2001) Differential subcellular distribution and transcriptional activity of Σ E3, Σ E4, and Σ E3–4 isoforms of the rat estrogen receptor- α . *Mol Endocrinol* 15:894–908
- Phi-Van L, Strätling WH (1988) The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO J* 7:655–664
- Sanders MM, McKnight GS (1985) Chicken egg white genes: multihormonal regulation in a primary cell culture system. *Mol Endocrinol* 116:398–405
- Sanders MM, McKnight GS (1988) Positive and negative regulatory elements control the steroid-responsive ovalbumin promoter. *Biochemistry* 27:6550–6557
- Shi Y, Lee J-S, Galvin KM (1997) Everything you have ever wanted to know about Yin Yang 1. *Biochim Biophys Acta* 1332:F49–F66
- Short ML, Nickel J, Schmitz A, Renkawitz R (1996) Lysozyme gene expression and regulation. *EXS* 75:243–257
- Spelsberg TC, Knowler JT, Moses HL (1974) Specific methods for the isolation of nuclei from chick oviduct. *Methods Enzymol* 31:263–279
- Steiner C, Muller M, Baniahmad A, Renkawitz R (1987) Lysozyme gene activity in chicken macrophages is controlled by positive and negative regulatory elements. *Nucl Acids Res* 15:4163–4178
- Sui G, Affar EB, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR, Shi Y (2004) Yin Yang 1 is a negative regulator of p53. *Cell* 117:859–872
- Thomas MJ, Seto E (1999) Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? *Gene* 236:197–208