Chicken vigilin gene: a distinctive pattern of hypersensitive sites is characteristic for its transcriptional activity

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Vigilin, a multidomain hn-ribonucleo-K-homologous protein, is part of a ribonucleoprotein complex with cognate tRNA and is found in both the nucleus and the cytoplasm. In an approach to identify genomic regions involved in regulation of the chicken vigilin gene, we carried out transfection studies with a reporter gene in suitable chicken cells. After including a distantly positioned 5'-sequence in the construct, we observed a 10.5-fold increase in luciferase (EC 1.13.12.7) expression compared with basal promoter activity. Accordingly, chromatin analysis of freshly isolated embryonic tendon fibroblasts with high levels of

vigilin mRNA expression shows a DNase-I-hypersensitive site (DHS1) localized 2.2 kb upstream of the transcriptional start site. Similarly, phytohaemagglutinin-stimulated lymphocytes with a 4-fold elevated expression of vigilin mRNA compared with resting lymphocytes also exhibited this unique DHS, having switched from that found at 3.3 kb (DHS2) in resting lymphocytes. Furthermore, using gel-retardation experiments with DNA representing either DHS1 or DHS2, a specific interaction with chicken nuclear extracts was seen.

INTRODUCTION

Vigilin, a 155 kDa protein ubiquitously found in vertebrates [1], contains 14 copies of the hn-ribonucleoprotein K-homologous motif, which is one of the major RNA-binding motifs [2,3]. Vigilin includes a nuclear localization sequence (NLS) between the hn-ribonucleoprotein K-homologous domains 2 and 3, and is localized in both the nucleus and the cytoplasm [4], the latter being associated with the rough endoplasmic reticulum [5]. Furthermore, vigilin is part of a ribonucleoprotein complex, which binds tRNA [6] and may thus play a functional role in the export of tRNA from the nucleus [6]. It is interesting to note that expression of vigilin is generally high both in established cell lines and in cells undergoing developmental differentiation [7].

Regulation of eukaryotic gene expression is associated with structural alterations of chromatin, which is seen in the exposure of DNase-I-hypersensitive sites (DHSs) [8,9]. These sites indicate promoter regions or *cis*-acting elements of active genes where transcription factors bind to regulate gene expression [10–12]. The activity of genes is also influenced by the extent of methylation, as hypomethylation facilitates binding of proteins to target sequences [13,14]. A large array of genes has been studied in recent years to identify mechanisms of gene regulation in eukaryotes. Appropriate regulation of gene expression has been shown to require activator proteins bound to regulatory elements [15,16], as well as mediating cofactors [17–19], which interact with the preinitiation complex at the promoter [20]. In addition, gene activation requires rearrangements in chromatin structure, mediated by changes in histone acetylation [21,22] and binding of the SWI–SNF multiprotein complex [23].

In previous studies the transcriptional start point and potential promoter elements (TATA and CAAT box) of the chicken vigilin gene had been identified [24], but the functional bases underlying the mechanisms of regulation were as yet unknown.

It was the aim of this study to demonstrate the functional activity of the promoter and to identify elements in the vigilin

gene responsible for the up-regulation of its expression. Along these lines we examined the 5'-region of the chicken vigilin gene by reporter-gene analysis and DHS mapping. The basal promoter activity was enhanced 10.5-fold by sequences localized upstream of the transcriptional start site. We have identified two segments of DNA sensitive to DNase I (EC 3.1.21.1), one exclusively observed under conditions of high transcriptional activity (DHS1) and the second restricted to conditions of low transcriptional activity (DHS2). Furthermore, we demonstrate specific binding of nuclear proteins to DHS1, providing evidence for its function as an enhancer element.

EXPERIMENTAL

Preparation of embryonic tendon fibroblasts

Tendons from 16-day chicken embryos were prepared under sterile conditions, washed twice with PBS and incubated on a shaker for 1 h at 37 °C in PBS containing 0.25 % (w/v) trypsin and 0.02 % (w/v) EDTA. Tendons were pelleted by centrifugation (1 min, 400 g). The supernatant containing the dissociated fibroblasts was supplemented with Dulbecco's minimal essential medium supplemented with 10 % (v/v) fetal-calf serum (Biochrom, Berlin, Germany). The tryptic digestion was repeated and the isolated tendon fibroblasts were cultured in Dulbecco's minimal essential medium supplemented with 10 % fetal-calf serum at 37 °C in air/CO₂ (19:1). Other eukaryotic cells used, such as CEC32 cells [25] or chicken embryonic chondrocytes [26], were cultured under the same conditions.

Preparation of lymphocytes

Freshly drawn blood from chicken was treated with heparin and diluted 1:2 with PBS, layered on top of lymphocyte separation medium (ICN, Meckenheim, Germany) and centrifuged for

Abbreviations used: CAT, chloramphenicol acetyltransferase; DHS, DNase-I-hypersensitive site; Dig, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LUC, luciferase; PHA, phytohaemagglutinin.

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30 min (400 g, 18 °C). The layer of lymphocytes was aspirated with a Pasteur pipette, washed with PBS and cultured in serum-free RPMI 1640 (Sigma, Deisenhofen, Germany). Lymphocytes were stimulated by adding phytohaemagglutinin (PHA) (50 µg/ml) to the culture medium.

Construction of luciferase expression vectors

Conventional DNA recombinant techniques for the construction of luciferase expression vectors were used according to standard protocols [27]. The promoter-deficient luciferase vector pWPLUC3 was constructed by replacing the chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) transcription unit of pBLCAT3 [28] by the luciferase (LUC) transcription unit of the vector pSV232AL-AA-5' [29]. The LUC transcription unit is composed of (a) the LUC-encoding DNA, which is a 5'- and 3'-deleted fusion product between crucial genomic DNA sequences and the respective cDNA from firefly *Photinus pyralis*, (b) an intron for the small T-antigen of SV40 and (c) an SV40 polyadenylation signal. The LUC transcription unit of pSV232AL-AA-5' was cleaved from the pBR322 vector by *Hind*III and *Bam*HI digestion. 5'-Overhanging ends were blunted with mung-bean nuclease (EC 3.1.30.1). The vector pBLCAT3 was depleted of the CAT transcription unit by digestion with *Bgl*II and *Sma*I and the 5'-overhanging ends were filled in with the Klenow fragment of DNA polymerase I (EC 2.7.7.7). The vector was dephosphorylated with calf intestinal alkaline phosphatase (EC 3.1.3.1) and ligated with T4 DNA polymerase to the luciferase transcription unit. As a consequence of this procedure, a *Bgl*II restriction site was reconstituted at the junction between the filled-in *Bgl*II site of the vector and the trimmed *Hind*III site of the LUC transcription unit.

Northern-blot analysis

Total RNA from various tissues of chicken embryos was prepared using guanidine/thiocyanate lysis and CsCl density step gradient centrifugation as described previously [30]. RNA was prepared from lymphocytes by acid guanidine thiocyanate/phenol/chloroform (1:1:0.2, by vol.) treatment [31]. RNA was separated on denaturing formaldehyde gels and blotted on to nylon membranes by the downward capillary transfer protocol [32]. Hybridization was performed using antisense RNA probes (Vig-c-670) labelled with digoxigenin (Dig), which were transcribed *in vitro* from part of the vigilin cDNA clone c-292 (from position 3451 to 4120) using the protocol of the manufacturer [33]. For immunological detection, CDP-*Star* (Boehringer, Mannheim, Germany) was used as chemiluminescent substrate for alkaline phosphatase. Hybridization and detection procedures were carried out by the protocol of the manufacturer (Boehringer). As an internal control for quantification of vigilin mRNA levels after stimulation of lymphocytes with PHA, a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antisense RNA was used. The amount of vigilin mRNA was corrected for the variation in the levels of the GAPDH content. Densitometric quantification was performed using whole-band scanning (Optoquant; Computer & Vision, Lübeck, Germany).

Transfection assays

A set of DNA fragments from the 5'-flanking region of the chicken vigilin gene was fused to the 5'-end of the luciferase gene of vector pWPLUC3. Analysed regions extended from -0.25 kb to +0.1 kb (p0.35+LUC), from -1.5 kb to +0.1 kb (p1.6+LUC), from -1.8 kb to +0.1 kb (p1.9+LUC) and from -7.8 kb to +0.1 kb (p7.9+LUC) relative to the transcription

initiation site. In all four subclones, the structural elements of the putative vigilin promoter were located at the same distance from the translation start of the luciferase gene. Another subclone comprised the vigilin 5'-flanking region in antisense orientation in front of the LUC gene (p8.1-LUC, from +0.3 kb to -7.8 kb).

Before transfection by electroporation, cells were trypsinized, washed with ice-cold PBS and adjusted to $(1-2) \times 10^7$ cells/ml in PBS. Plasmid DNA (10 µl, equivalent to 1-3 µg of test-LUC plasmid and 1-3 µg of pBLCAT2 [28] DNA) were mixed with 90 µl of cells, which were electroporated at 750-1750 V/cm and 25 µF, corresponding to a time constant of 2.1-3.1 ms. After electroporation (48 h) cells were lysed in 250 µl of lysis buffer [25 mM Tris/phosphate (pH 7.8)/1 mM dithiothreitol/1 mM EDTA/1% (v/v) Triton X-100/1% (w/v) BSA/15% (w/v) glycerol]. CAT activity for normalization was measured by a modified fluor-diffusion CAT assay [34]. LUC activity was measured using a liquid-scintillation counter in the single-photon count modus [35], with coenzyme A and dithiothreitol for the stabilization of light emission [36].

Mapping of DHSs

Mapping of DHSs was performed according to the protocol of Dirks et al. [37]. Cells (1×10^8) were grown to subconfluency in 150 mm $\times$ 25 mm tissue-culture dishes (Becton-Dickinson, Toronto, Canada), washed twice with cold PBS and scraped off the dishes with a cell lifter. All the following steps were performed at 4 °C. Pelleted cells (5 min, 600 g) were resuspended and lysed in lysis buffer [0.15 mM spermine/0.5 mM spermidine/15 mM Tris/HCl (pH 7.4)/60 mM KCl/15 mM NaCl/2 mM EDTA/0.5 mM EGTA/500 mM sucrose/1 mM Pefabloc/0.35% (v/v) Nonidet P40] by six strokes in a pestle-B Dounce homogenizer (Wheaton, Millville, NJ, U.S.A.). Pelleted nuclei (5 min, 1000 g) were washed three times in washing buffer (lysis buffer without Nonidet P40 but with 350 mM sucrose) and resuspended in digestion buffer [0.15 mM spermine/0.5 mM spermidine/15 mM Tris/HCl (pH 7.4)/60 mM KCl/15 mM NaCl/0.2 mM EDTA/0.2 mM EGTA] at a concentration of 2×10^8 nuclei/ml. DNase I digestions were performed at room temperature in volumes of 100 µl with 2×10^7 nuclei and increasing concentrations (0-80 units) of DNase I (Boehringer). Reactions were started by addition of 5 mM MgCl₂ and stopped after 5 min by addition of 10 mM EDTA. Nuclei were resuspended in 1 ml of buffer G2 from Qiagen (Hilden, Germany) [800 mM guanidinium chloride/30 mM EDTA/30 mM Tris/HCl/5% (v/v) Tween 20/0.5% Triton X-100 (pH 8.0)] supplemented with 300 µg of proteinase K (EC 3.4.21.64), and were incubated at 50 °C for 30 min. Genomic DNA was isolated with a genomic DNA purification kit (Qiagen), and purified DNA (20 µg samples) was then digested with *Eco*RI (New England Biolabs, Schwalbach, Germany) for 3 h at 37 °C. DNA samples were electrophoresed in horizontal 0.8% (w/v) Tris/acetate/EDTA-agarose gels and transferred to Hybond N⁺ (Amersham, Braunschweig, Germany) or BM membranes (Boehringer) by downward capillary transfer [32]. DNA was hybridized overnight with a Dig-labelled or a ³²P-labelled RNA probe (p250, an *Eco*RI-*Bgl*II fragment ranging from +0.3 to +0.1 kb of the chicken vigilin gene) under stringent conditions [30,33]. For radioactive blots, X-ray film (Fuji, Tokyo) exposure was performed at -80 °C overnight or for several days using an intensifying screen. Film exposure was limited to 1-10 min in those instances where Dig-based immunological detection was used.

Electrophoretic mobility-shift assay

A nuclear extract from CEC32 cells [25] was prepared according to Dignam et al. [38]. DNA fragments (10 pmol) amplified by PCR from the DHS regions were end-labelled using T4 polynucleotide kinase and [γ - 32 P]ATP (3000 Ci/mmol). Nuclear extract (10 μ g) and sheared fish sperm DNA (60 μ g), as non-specific competitor, were preincubated in binding buffer [250 mM glycine (pH 7.5)/100 mM NH_4Cl /50 mM MgCl_2 /25 mM dithioerythritol/1 mM EDTA] in 30 μ l total volume for 10 min on ice. Then radioactively labelled DNA (20000 c.p.m.) was added and incubation continued for an additional 15 min at room temperature. In competition assays, a 100-fold excess of unlabelled DNA ($\approx$ 1.9 pmol), amplified from the same region, was used to demonstrate the specificity of the gel shift. The DNA-protein complexes were identified by separation of the free probe from the DNA-protein complex in a 4% (w/v) native polyacrylamide gel, with 0.5 $\times$ 45 mM Tris/borate/1 mM EDTA, pH 8.0 as running buffer.

RESULTS

Expression of the chicken vigilin gene in embryonic cells

Previous studies have shown increased vigilin expression during chick embryonic development. Highest steady-state levels of vigilin mRNA were observed during developmental stages known for their high rates of protein synthesis, such as cartilage and bone formation [7]. Here we analysed the occurrence of vigilin in different cells isolated from 16-day chicken embryos by Northern-blot analysis in order to find embryonic cells suitable as an autologous system for transient transfection experiments.

As shown in Figure 1, each of the four different tissues showed a substantial amount of vigilin mRNA, being most abundant in sterna and tendon. Thus chondrocytes from sterna and fibroblasts from tendon appeared to be suited best to analyse the 5'-flanking region of the chicken vigilin gene under conditions that should ensure high levels of reporter gene expression. In subsequent

Figure 1 Northern-blot analysis of different tissues isolated from 16-day-old chicken embryos

Total RNA was separated on a denaturing formaldehyde-agarose gel, blotted on to a nylon membrane and hybridized with the chicken vigilin-gene-specific and Dig-labelled antisense RNA probe Vig-c-670. Detection of the bound probe was achieved with the chemiluminescent reagent CDP-Star. (a) The blot shows two vigilin-mRNA-specific signals (indicated on the left) due to polyadenylation at different 3'-sites [48]. Nucleotide markers are shown on the right. (b) As an internal control for the amount of RNA loaded on to each lane, the 18 S rRNA stained with ethidium bromide is shown by UV transillumination.

Figure 2 Constructs used in reporter-gene assays

(a) Physical map of the region of the vigilin gene analysed in transient transfection assays. Distances (in kb) of several restriction sites relative to the transcription initiation site are indicated. (b) Different vigilin-LUC plasmid constructs are represented and shown by their length of 5'-flanking regions.

Table 1 Effect of truncations in the 5'-flanking region of the vigilin gene on reporter-gene expression in embryonic chicken-tendon fibroblasts

Relative levels of reporter-gene expression in primary embryonic chicken-tendon fibroblasts transfected by electroporation. LUC expression levels obtained with the different constructs were compared with that of p0.35+LUC (basal promoter activity). Values are means $\pm$ S.E.M. of three independent transient transfection assays, and were normalized to the CAT activity after co-transfection with the CAT expression vector pBLCAT2.

LUC reporter plasmid	LUC activity (fold)
p0.35 + LUC (−0.25 kb to +0.1 kb)	1.0 $\pm$ 0.2
p1.6 + LUC (−1.5 kb to +0.1 kb)	0.2 $\pm$ 0.06
p1.9 + LUC (−1.8 kb to +0.1 kb)	0.3 $\pm$ 0.04
p7.9 + LUC (−7.8 kb to +0.1 kb)	10.5 $\pm$ 0.5
p8.1 − LUC (+0.3 kb to −7.8 kb)	0.01 $\pm$ 0.005

transient transfection experiments, however, it became evident that the transfection of chondrocytes by electroporation was less efficient than that of fibroblasts, which yielded very high levels of reporter gene expression (results not shown), leaving the latter as the autologous system of choice.

Functional identification of the vigilin promoter: basal transcriptional activity is modulated by distant *cis*-acting elements

Previous studies of the chicken vigilin gene have revealed a distinct transcription initiation site, as well as consensus sequences characteristic for eukaryotic promoter elements [24]. Therefore the 5'-region of the chicken vigilin gene was studied in the range from +0.1 kb to −7.8 kb for its ability to drive transcription of the reporter gene luciferase in transiently transfected embryonic chicken fibroblasts.

Figure 2 is a schematic diagram of the analysed region of the vigilin gene and the LUC-reporter constructs used. The numbers assign the position relative to the transcription start site. Table 1 shows the tested constructs along with their relative LUC activities. For comparison we defined the LUC activity obtained after transfection of the LUC reporter plasmid p0.35+LUC as 1. This region between −0.25 kb and +0.1 kb represents the basal activity of the vigilin promoter (Table 1). When additional 5'-flanking sequences up to −7.8 kb were investigated

Figure 3 Identification of DHS in the 5'-region of the chicken vigilin gene

DNase-I-treated genomic DNA from embryonic tendon fibroblasts was digested with *EcoRI*, separated on a 0.8% agarose gel, blotted and hybridized with the ^{32}P -labelled probe p250. Embryonic tendon fibroblasts with high levels of vigilin expression show a DHS at 2.5 kb (DHS1), indicated on the right. The distance to the transcriptional start point is 2.2 kb (see Figure 5).

Table 2 Quantification of vigilin mRNA-levels in PHA-stimulated lymphocytes

Densitometric analysis revealed a 4-fold increase in the level of vigilin mRNA after 48 h of stimulation with PHA. Values were normalized to GAPDH signals. Results are means $\pm$ S.E.M. for three independent experiments.

Time after PHA stimulation (h)	mRNA signal (% of maximum)
0	24.5 $\pm$ 1.3
12	35.5 $\pm$ 2.8
28	34.2 $\pm$ 3.1
48	100

(p1.6+LUC), the basal transcription activity was decreased by 5-fold. A further 0.3 kb extension into the 5'-flanking region (p1.9+LUC) did not significantly change the influence of the silencing region located between -0.25 kb and -1.5 kb (p1.6+LUC). After transfection of embryonic chicken fibroblasts with the LUC-reporter plasmid p7.9+LUC (containing the 5'-region of the vigilin gene from -7.9 kb to $+0.1$ kb), the silencing activity was bypassed and the resulting transcriptional activity was approx. 10.5-fold higher than the activity seen after transfection with p0.35+LUC (basal activity). Background activity was determined following transfection with the construct p8.1+LUC, which encompasses the presumed promoter region and the adjacent 5'-region, though in opposite orientation. This construct exhibited only 0.01-fold of the activity seen in cells after transfection with p0.35+LUC, where the tested region is in the functional orientation relative to the reporter gene.

Mapping of DHS in embryonic tendon fibroblasts and in chicken lymphocytes

To define structurally the region of potential *cis*-acting elements, which are essential for the silencing and enhancing effects described in Figure 2, the chromatin of intact nuclei isolated from embryonic tendon fibroblasts was analysed for DHSs in the 5'-region of the vigilin gene. As shown in Figure 3, the tendon fibroblasts show a DHS at 2.5 kb (DHS1), i.e. 2.2 kb upstream of the transcriptional start point (compare Figure 5b), indicating

Figure 4 Northern-blot analysis of PHA-stimulated lymphocytes isolated from chicken blood

Total RNA was prepared at the indicated times after PHA stimulation and aliquots were separated on a denaturing formaldehyde-agarose gel, blotted on to a nylon membrane and hybridized with a chicken vigilin-specific and Dig-labelled antisense RNA probe that was prepared as described in the Experimental section. A human GAPDH-specific antisense RNA probe was used as a control. For detection of the bound probe the chemiluminescent reagent CDP-Star was employed. The autoradiogram is representative of three independent experiments that gave similar results.

the occurrence of regulatory elements in this region of the gene. A second set of experiments was based on the observation that human lymphocytes with low levels of vigilin can be stimulated by PHA to express high levels of vigilin [1]. Under these conditions we analysed resting lymphocytes isolated from chicken blood after incubation with PHA. As shown in Table 2 and Figure 4, levels of vigilin mRNA remained on a basal plateau for approx. 28 h, whereas a 4-fold increased level was observed after 48 h of incubation. Accordingly, the chromatin of unstimulated lymphocytes shows a DHS at 3.6 kb (DHS2), i.e. 3.3 kb upstream of the transcriptional start site (Figure 5a). After 48 h of stimulation with PHA, however, a switch in hypersensitivity of the chicken chromatin to 2.5 kb became apparent. This DHS is localized in a position identical with that found in embryonic tendon fibroblasts (see Figure 3). DNase I mapping of the chromatin from whole blood cells isolated from chicken serum confirms the DHS2 at 3.6 kb, the same as that observed for unstimulated lymphocytes (Figure 5a). The shift of the DHS, which is correlated with high steady-state levels of vigilin mRNA, confirms the existence of an enhancer element in the region of DHS1 at -2.5 kb.

Electrophoretic mobility-shift analysis with DNA fragments representing DHS1 and DHS2

Four DNA fragments comprising the detected hypersensitive sites DHS1 and DHS2 were analysed for their binding to nuclear proteins (Figure 6a). Positions of the DNA fragments relative to the transcriptional start point are indicated in Figure 6(a): p2/201, extending from -2097 bp to -1896 bp, and p3/240, extending from -3241 bp to -3001 bp, showed no protein binding when incubated with a nuclear extract prepared from the chicken fibroblast cell line CEC32 under the separation conditions applied (results not shown). Both fragments do not match the mapped DHS completely and were used as controls. Retardation assays with fragments p1/174 (position -3418 bp to -3244 bp) and p4/213 (position -2293 bp to -2080 bp), both of which fit the respective DHS (Figure 6b), showed a shift with the nuclear extract in the presence of $60 \mu\text{g}$ of sheared fish sperm DNA as non-specific competitor. The shift could be competed by the addition of a 100-fold excess of unlabelled p1/174 or p4/213, indicating specific interaction of nuclear proteins with these DNA regions (Figure 6b).

Figure 5 Identification of DHS in chicken lymphocytes

(a) DNase-I-treated genomic DNAs from chicken lymphocytes, PHA-stimulated chicken lymphocytes and chicken blood cells were digested with *EcoRI*, separated on 0.8% agarose gel, blotted and hybridized with Dig-labelled (unstimulated lymphocytes) or ^{32}P -labelled (PHA-stimulated lymphocytes and chicken blood cells) probe p250. In chicken blood cells and unstimulated lymphocytes a hypersensitive region is detected at 3.6 kb (as shown by the arrows). PHA-stimulated lymphocytes with a 4-fold increase in vigilin mRNA levels show distinct DHS at 2.5 kb (also shown by an arrow). (b) Physical map of the vigilin gene and positions of DHS1 and DHS2 relative to the transcriptional start point. The positions of the probe p250 and a GC-rich region between DHS1 and DHS2 are also indicated.

DISCUSSION

In an approach to understand how cells activate vigilin expression at differential rates, we used embryonic mesenchymal cells with high steady-state levels of vigilin mRNA in parallel with lymphocytes that can be raised to high levels of vigilin mRNA following stimulation with PHA. The aim of the study was to identify regulatory elements at the 5'-flanking region that would drive the promoter activity of the vigilin gene. Transient transfection with a construct comprising all 8 kb of the 5'-flanking sequence ($p7.9+\text{LUC}$) enhanced the basal promoter activity 10.5-fold, whereas 5'-flanking sequences up to -1.5 kb decreased the basal transcription by 5-fold. These data suggest that *cis*-acting elements located beyond -1.5 kb of the transcriptional start site up-regulate the expression of the vigilin gene. Based on these observations we extended our study so as to identify for the first time *cis*-acting elements responsible for the enhancing activity by analysing the chromatin harbouring the chicken vigilin gene with respect to its sensitivity to DNase I. We identified a hypersensitive site (DHS1) that was common to both embryonic mesenchymal cells and lymphocytes following stimulation with PHA. DHS2, the second hypersensitive site, was observed only in unstimulated lymphocytes with a low level of vigilin expression. The occurrence of DHS1 only in cells with high vigilin expression clearly indicates that an enhancer element might be present in this region. No structural evidence is available at present that could explain the silencing effects residing in a region up to -1.5 kb upstream of the transcriptional start point. In line with the preceding experiments, gel-retardation assays

demonstrated that DNA fragments representing DHS1 and DHS2 specifically interact with nuclear proteins. The binding of transcription factors to DHS1 might be required for gene activation, whereas the binding to DHS2 might be responsible for the low level of vigilin expression. In this context it is interesting to note that Abruzzo and Reitman [39] found four DHSs in the 5'-region of the β -globin gene, but only two of them correlated with an enhancer activity.

Sequence analysis of DHS1 and DHS2 for putative binding sites of *trans*-acting factors [40] revealed a large array of binding sites for different factors. Among the most prominent ones were three consensus sequences for putative binding of the transcription factor AP4 in DHS2 and a cluster of nine consensus sequences for AP4 in DHS1 (Figure 7). AP4 is an enhancer binding factor isolated from HeLa-cell nuclear extracts that activates SV40 late transcription *in vitro* and belongs to the helix-loop-helix proteins [41].

Enhancer elements containing AP4 binding sites are found in a variety of genes such as the β -myosin heavy-chain enhancer [42] or the human transferrin gene enhancer [43]. Most noteworthy is the occurrence of AP4 binding sites in genes expressed by exocrine pancreatic cells such as the pancreas elastase gene [44] or the α -amylase 2A transcription enhancer [45]. In line with this notion, it has been shown that in rat exocrine pancreas, as well as in rat endometrial cells, induction of vigilin expression depends on stimulation by appropriate exposure to hormones (oestrogen/progesterone) or feeding, and is correlated in the pancreas with secretion of digestive enzymes [5,46]. In either instance, enhanced vigilin expression levels are associated with morphological

Figure 7 Distribution of AP4 binding sites in the 5'-region of the chicken vigilin gene

(a) Overall distribution of AP4 binding sites in the 5'-region of the chicken vigilin gene. DHS1, DHS2 and AP4 binding sites are indicated. (b) Location of AP4 binding sites in DHS1 (represented by the shifted DNA fragment p4/213) and DHS2 (represented by the shifted DNA fragment p1/174).

Based on the assumption that the gene regulatory mechanisms in rat and chicken might follow similar pathways, it is reasonable to assume that the influence of hormones on vigilin may derive from mechanisms that are not dependent on the presence of hormone-responsive elements, as they have been found neither in DHS1 nor in DHS2. Other regulatory factors most probably modulate vigilin expression directly by binding to the 5' upstream region. We have functionally identified these regions by transient transfections, DHS mapping and gel-retardation assays, although the protein characteristics of the *trans*-acting factors remain to be shown.

Interestingly, the promoter regions of the chicken and the human vigilin genes are completely different from each other, whereas the vigilin expression patterns in both species are rather similar [1,47]. The chicken vigilin gene promoter contains the typical structural features of an inducible promoter with a TATA and a CAAT box at appropriate distances from the transcriptional start point. The promoter of the human gene shows features characteristic of a housekeeping-gene promoter, such as high GC content and putative binding sites for SP1. Additionally, the human gene contains two 5' exons [47] that are not present in the chicken gene, which may play a role in both transcriptional and translational regulation.

In summary, we have shown that a switch of DHS occurs in the chromatin of the chicken vigilin gene that is paralleled by the activation of an enhancer element containing clustered sites for putative binding of AP4 and other transcription factors. Further

Figure 6 Gel-retardation assays with DNA fragments comprising DHS1 and DHS2

(a) Positions of the four DNA fragments relative to DHS1 and DHS2. The GC content between the two indicated *KpnI* sites is approx. 90%. (b) A specific shift with a nuclear (nuc.) extract isolated from CEC32 cells was observed when using DNA fragments p1/174 and p4/213 as probes in the presence of 60 µg of sheared fish sperm DNA as non-specific competitor. A 100-fold excess of unlabelled p1/174 and p4/213 was used for specific competition. No complex-formation was observed when probes p3/240 and p2/201 were incubated with the nuclear extract under the separation conditions used.

features that are characteristic of high protein synthesis, such as an expanded rough endoplasmic reticulum and an increase in the number of secretory granules [46].

studies will be required to elucidate the regulation of the chicken vigilin gene and to identify the transcription factors involved.

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