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Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family

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Deposition of the yolk mass components of chicken oocytes, very low density lipoprotein (VLDL) and vitellogenin (VTG), is mediated by a 95 kDa plasma membrane protein, termed VLDL/VTG receptor (VLDL/VTGR). Molecular characterization of the VLDL/VTGR revealed that it is a member of the LDLR gene superfamily, and harbours eight complement-type, cysteine-rich ligand binding repeats at the N-terminus. This ligand binding domain structure is the hallmark of the recently discovered mammalian so-called VLDLRs, whose true physiological function remains to be elucidated. Northern blot analysis revealed that this receptor is expressed almost exclusively in oocytes, with very much lower levels of hybridizing transcripts present in heart and skeletal muscle. Heterologous expression of the cloned receptor demonstrated its ability to bind both VLDL and VTG. The receptor gene is located on the avian sex chromosome Z, in agreement with the sex linkage of a single-gene defect in animals that fail to reproduce because of the lack of expression of functional VLDL/VTGR. *In situ* hybridization analysis of oocytes suggested that VLDL/VTGR mRNA may relocalize during oocyte growth. Thus, the current study has identified and characterized the first non-mammalian VLDLR. Its key role in avian reproduction and extremely high evolutionary conservation shed new light on VLDLR function in mammals, which also express the gene in ovaries.

Key words: avian/oocyte/receptor/reproduction/vitellogenesis

Introduction

Recent cloning efforts in several laboratories (Herz *et al.*, 1988; Raychowdhury *et al.*, 1989; Takahashi *et al.*, 1992; Yochem and Greenwald, 1993; Gåfvels *et al.*, 1993; Oka *et al.*, 1994; Nimpf *et al.*, 1994; Sakai *et al.*, 1994; Webb *et al.*, 1994) have identified an ever increasing number of relatives of the mammalian low density lipoprotein receptor (LDLR). Probably the most fascinating aspect of this gene family is that the physiological roles of its members appear to be quite diverse as reflected by a wide range of ligands (for a recent review, see Schneider and Nimpf, 1993), although they have several structural elements in common.

These common structural modules are (i) the so-called 'binding repeats', complement-type domains consisting of ~40 residues displaying a triple-disulfide-bond-stabilized negatively charged surface. Certain head-to-tail combinations of these repeats are believed to specify ligand interaction; (ii) epidermal growth factor precursor-type repeats, also containing six cysteines each; (iii) modules of ~50 residues with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD); and (iv), in the cytoplasmic region, signals for receptor internalization via coated pits, containing the consensus tetrapeptide Asn-Pro-Xaa-Tyr (NPXY). The best characterized binding domain is that of the LDLR, which consists of seven ligand binding repeats and recognizes apoB and apoE (Russell *et al.*, 1989). Both naturally occurring and site-specifically introduced mutations have defined the minimal requirements for recognition of ligands via either of the two apolipoproteins (reviewed in Schneider, 1989).

Recently, a receptor with a single cluster of eight binding repeats has been added to the list of LDLR gene family members (Takahashi *et al.*, 1992). The hitherto identified preferred ligand of this receptor is very low density lipoprotein (VLDL) rather than LDL, hence its designation VLDLR. The structural basis for preferential recognition of VLDL, in particular the role of the extra N-terminal ligand binding repeat relative to the LDLR, has not been investigated.

LDLR family genes are often co-expressed in a variety of tissues and cells (Kerjaschki and Farquhar, 1982; Kowal *et al.*, 1989; Stifani *et al.*, 1991; Gåfvels *et al.*, 1993; Sakai *et al.*, 1994; Webb *et al.*, 1994) with different metabolic requirements and functions. Thus, there are at least three major groups of questions in the field of LDLR family members awaiting definitive answers. First, given the extensively overlapping ligand recognition and expression patterns of these receptors, how does each of them perform its individual task(s) in the presence of several similar membrane proteins? Second, what are the modes of regulation of expression of the individual receptor genes? Third, are there any additional as yet undiscovered members of the LDLR family?

Our studies on the LDLR gene family of the chicken, particularly in the laying hen, have begun to address these questions, with particular emphasis on cell type-specific receptor gene expression and function. The aspects amenable to investigation in the laying hen result from the unique physiological challenges posed by egg-laying, coupled to an otherwise typical network of receptor-mediated systemic transport pathways. Studies in our laboratory to date have revealed that in order to meet

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these challenges, the laying hen expresses two pairs of related genes belonging to the LDLR family. We have identified two proteins that function in systemic homeostasis via transport of ligands of the LDLR family: a 130 kDa LDLR (Hayashi *et al.*, 1989) and a 600 kDa LRP (Stifani *et al.*, 1991; Nimpf *et al.*, 1994). These proteins are absent from oocytes; however, each has a partner which is abundant in growing oocytes, but apparently absent from somatic tissues. These are (i) a 95 kDa protein that binds VLDL and VTG (the VLDL/VTG receptor), whose absence leads to the failure of oocyte growth and consequently the failure to produce offspring (Nimpf *et al.*, 1989), and (ii) an LRP of ~380 kDa (Stifani *et al.*, 1991). Thus, the laying hen has at its disposal at least four receptor proteins known to be the products of four different related genes (Barber *et al.*, 1991; Stifani *et al.*, 1991; Nimpf *et al.*, 1994), providing an exciting scenario to unravel the molecular basis for differentiated function and cell-specific expression of LDLR gene family members in the context of a well-defined physiological background.

In order to facilitate the delineation of molecular details underlying dichotomy of the LDLR pathway in the laying hen, we now report new information relating to the abundant oocytic 95 kDa protein. Surprisingly, its ligand binding domain consists of eight, not seven, ligand binding repeats, and thus it is the chicken homologue of mammalian VLDL receptors. Since this receptor is pivotal to oocyte growth, i.e. to avian reproduction, it will be of particular interest to correlate the current findings with the proposed function(s) of VLDLRs in mammals, which also appear to express this or a closely related receptor in ovarian tissue (Webb *et al.*, 1994).

Results

The chicken VLDL/VTG receptor cDNA and protein

In order to identify a chicken VLDL/VTGR cDNA by the polymerase chain reaction (PCR), two degenerate oligonucleotides (17-mers) corresponding to tryptic peptide sequences of the purified receptor (Barber *et al.*, 1991) were designed and synthesized. Using these degenerate oligonucleotides as primers to amplify cDNAs prepared from poly(A)$^+$ RNA of mature chicken ovaries, a ~0.8 kb PCR fragment, C8-1, was obtained, subcloned into a plasmid vector, and further analysed. The deduced amino acid sequence of C8-1 indicated that it was highly homologous to VLDL and LDL receptors. C8-1 was then used to probe a chicken ovary cDNA library. The screening resulted in the identification of a 3.3 kb cDNA insert, CVR-1, encoding the entire VLDL/VTGR sequence (Figure 1).

Sequencing of CVR-1 defined an open reading frame of 2589 bp (coding for 863 amino acids). A single ATG (methionine) codon is present in the 5′ part of the open reading frame, followed by a stretch of hydrophobic amino acid residues that presumably function as a cleavable signal sequence. Moreover, the ATG codon fulfilled the rules for translation initiation (Kozak, 1984), and therefore probably represents the translation initiation site. The preferred cleavage site for the signal peptidase, 44 residues downstream, was predicted according to von Heijne *et al.* (1983). Another hydrophobic region, presumably representing a putative transmembrane domain, is found at amino acid residues 744–765. The putative extracellular domain has a cysteine-rich region (see below) with two potential N-glycosylation sites. The calculated molecular weight of the mature protein is 90 230, in good agreement with the apparent M_r of 95 000 of the purified VLDL/VTGR protein determined by non-reducing SDS−PAGE (Barber *et al.*, 1991). The deduced amino acid sequence of CVR-1 contains all known tryptic peptide sequences of the isolated VLDL/VTGR (amino acids 466–474, 502–510 and 775–783, indicated in Figure 1) (Barber *et al.*, 1991).

Alignment of the amino acid sequence of the chicken VLDL/VTGR with those of the rabbit VLDLR and LDLR (Figure 2), the human VLDLR and LDLR and other LDLRs (Yamamoto *et al.*, 1984; Mehta *et al.*, 1991; Gåfvels *et al.*, 1993; Hoffer *et al.*, 1993; Oka *et al.*, 1994; Sakai *et al.*, 1994; Webb *et al.*, 1994) (not shown) suggests that the chicken VLDL/VTGR is, in fact, a homologue of the mammalian VLDL receptor. This notion is based on the presence of eight ligand binding repeats at the N-terminus (1–8 in Figure 2); the cluster of eight rather than seven such repeats, as present in LDLRs, is the signature of all VLDLRs characterized to date (Takahashi *et al.*, 1992; Gåfvels *et al.*, 1993; Oka *et al.*, 1994; Sakai *et al.*, 1994; Webb *et al.*, 1994). Furthermore, as in other LDLRs and VLDLRs, the C-terminal three repeats are separated by a 'linker' region from the N-terminal four (in LDLRs) or five (in VLDLRs) repeats (Figure 2). All cysteine residues in the ligand binding domains of LDLRs, the chicken oocyte receptor and VLDLRs are in identical positions. The eight repeats are followed by an epidermal growth factor (EGF) precursor homology domain (A−C in Figure 2), a putative transmembrane region, and a C-terminal domain of 54 amino acid residues; all other VLDLRs and LDLRs harbour these domains. Remarkably, despite the evolutionary distance between chicken and rabbit, the amino acid sequences of their VLDLRs show extremely high conservation. Identical residues are present in 93% of the positions in the cytoplasmic domains, 85% in the EGF precursor homology domains, and 84% in the ligand binding domains. Even the membrane spanning domain, generally the least conserved region in the LDLR gene family (Mehta *et al.*, 1991), shows 64% identity. As Figure 2 shows, the identity between rabbit and chicken eight-repeat receptors is much greater than that between LDL and VLDL receptors of the rabbit (this is also true for chicken versus human; data not shown).

However, in contrast to the rabbit VLDLR, the chicken oocyte VLDL/VTGR lacks a serine- and threonine-rich domain that is likely to carry clustered O-linked carbohydrate groups. When present, the O-linked sugar domains of members of the LDLR gene family are highly variable in length and sequence (e.g. only 20% identity between rabbit VLDLR and LDLR, Figure 2; Mehta *et al.*, 1991; Takahashi *et al.*, 1992). In addition, splice variants of the human VLDLR mRNA specifying two putative forms of VLDLR, with or without this domain, have been identified (Sakai *et al.*, 1994; Webb *et al.*, 1994). In the chicken, preliminary PCR-based experiments also suggest the presence of a VLDLR mRNA encoding an O-linked sugar domain-containing receptor different from that in oocytes (H.Bujo, unpublished observations). Five repeats

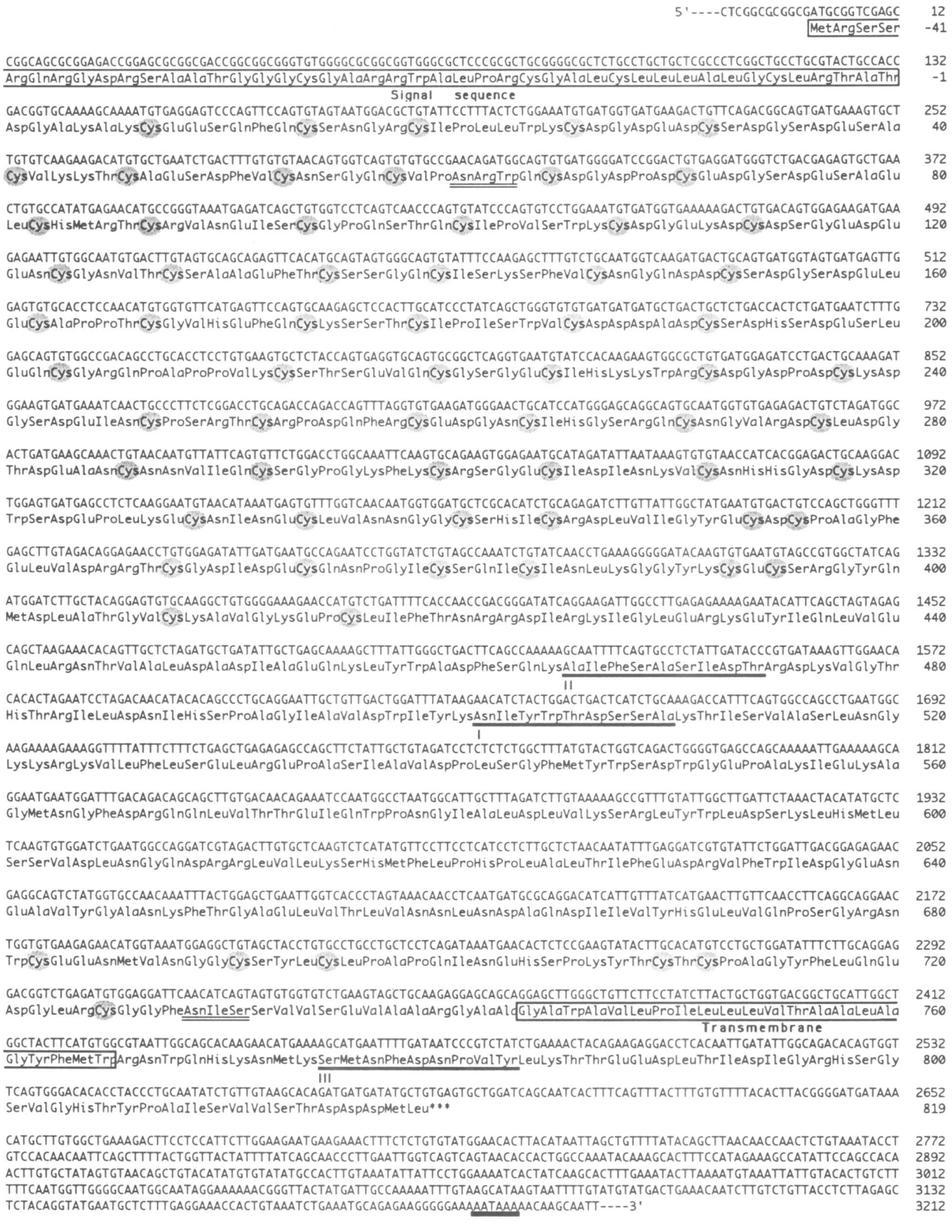


Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA encoding chicken VLDL/VTG receptor. The nucleotide sequence of the chicken VLDL/VTG receptor, together with the deduced amino acid sequence, is shown. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG triplet encoding the initiating methionine (Kozak, 1984), and negative numbers referring to the 5′-untranslated region. The amino acid sequence is numbered from the putative signal sequence cleavage site (von Heijne, 1983) and negative numbers refer to the cleavage sequence (boxed at the N-terminus). Three tryptic peptide sequences (I, II and III) obtained previously by microsequencing of the purified protein (Barber *et al.*, 1991) are underlined. Cysteine residues are shadowed. Two potential N-linked glycosylation sites are doubly underlined. The transmembrane segment located towards the C-terminus of the protein is boxed. Stop codon and potential polyadenylation signal are indicated by asterisks and a heavy underline, respectively. The EMBL Nucleotide Sequence Databank accession number for SSVLDLRCR is X80207.

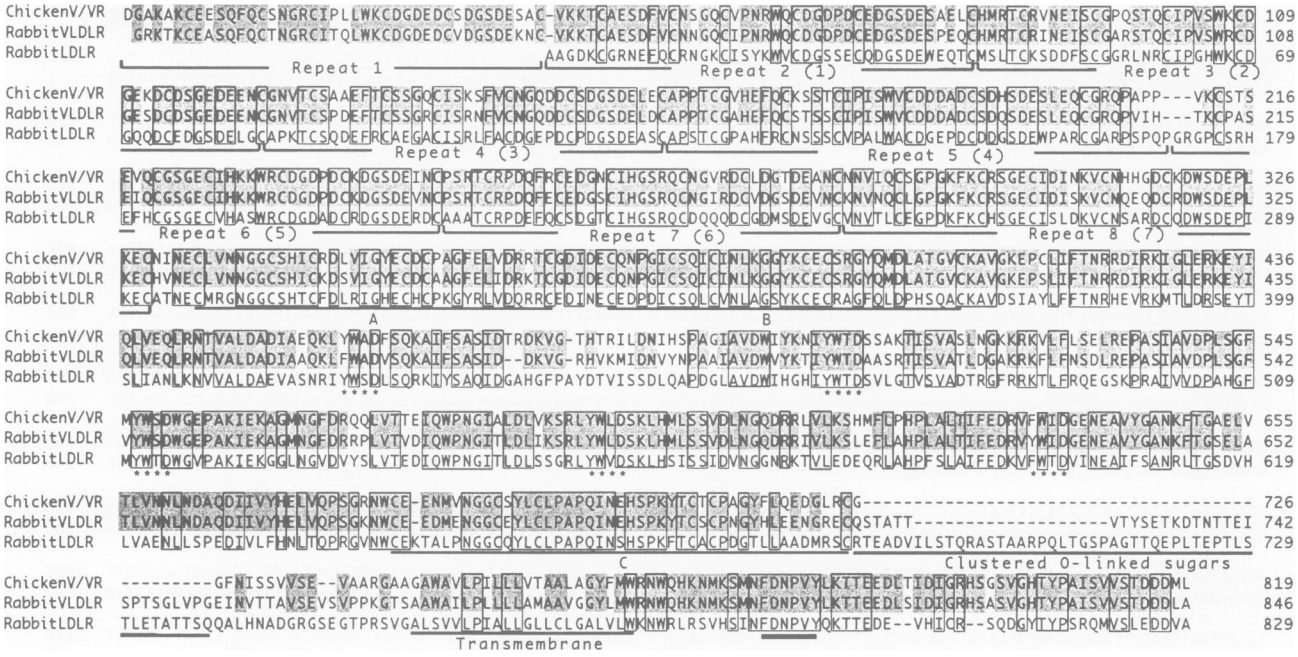


Fig. 2. Comparison of the amino acid sequences of the chicken VLDL/VTG receptor, the rabbit VLDL receptor (Takahashi *et al.*, 1992) and the rabbit LDL receptor (Yamamoto *et al.*, 1986). The amino acids are numbered from the N-terminus of the putative mature protein of each receptor. Gaps have been introduced to optimize the alignment. Amino acids identical in the chicken VLDL/VTG receptor and the rabbit VLDL receptor, and among three receptors including the rabbit LDL receptor are noted by shading with light gray and boxed, respectively. The ~40 amino acid repeats 1–8 (1–7 of the LDL receptor) in the ligand binding domains, cysteine-rich repeats A, B and C in the EGF precursor homology domains, clustered O-linked sugar regions and transmembrane domains are shown. The YWTD sequences are indicated by asterisks, and the FDNPVY sequence, required for clustering of the LDL receptor in coated pits (Chen *et al.*, 1990), is indicated by a heavy underline. V/V receptor, VLDL/VTG receptor.

containing a signature tetrapeptide (indicated by asterisks in Figure 2) between repeats B and C of the EGF precursor homology domain, and the internalization signal (Phe-Asp-Asn-Pro-Val-Tyr) (Chen *et al.*, 1990) are conserved in the chicken VLDL/VTGR (Figure 2).

Expression and immunological identification of the chicken VLDL/VTG receptor

The expression plasmid pCDMCVR-1 was prepared and used to transfect COS-7 cells. In order to test whether pCDMCVR-1 indeed leads to the expression of the same receptor that is present in chicken oocytes, we used two polyclonal antibodies in immunoblots of detergent extracts from oocyte membranes and transfected COS-7 cells (Figure 3). One of the newly raised antibodies, raised against purified VLDL/VTGR, reacts with the 95 kDa protein as well as another protein in oocyte extracts previously shown (Stifani *et al.*, 1991) to represent an oocyte-specific LRP (~380 kDa, Figure 3, lane 1). COS-7 cells transfected with the CVR-1-containing plasmid, but not with the vector alone, expressed a single cross-reactive 95 kDa protein co-migrating with the smaller oocyte protein (Figure 3, lanes 1–3). The other antibody, raised against an oligopeptide corresponding to the C-terminal 14 residues of the cloned receptor, reacted with the product of pCDMCVR-1 expression in COS-7 cells (lane 5); and importantly, also with the bona fide receptor of oocytes (lane 4). This antibody shows no crossreactivity with the oocytic LRP, nor with any other protein in chicken oocytes (lane 4); in COS-7 cells transfected with pCDMCVR-1 (lane 5) or vector alone (lane 6), there is a weakly crossreactive large protein, possibly a simian member of the LDLR gene family. These results demonstrate unambiguously that the cloned chicken cDNA encodes the previously described oocyte-specific VLDL/VTG receptor (George *et al.*, 1987; Steyrer *et al.*, 1990; Barber *et al.*, 1991).

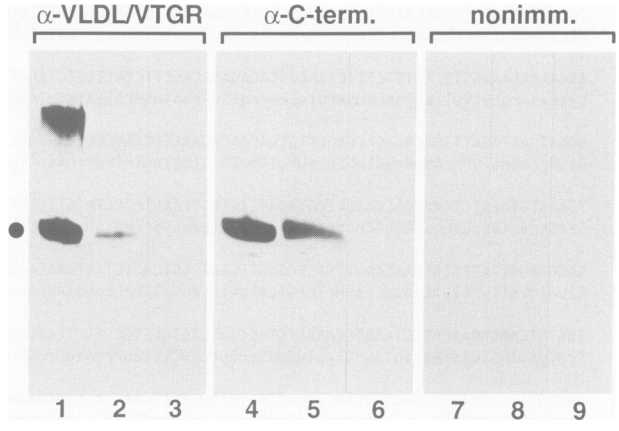
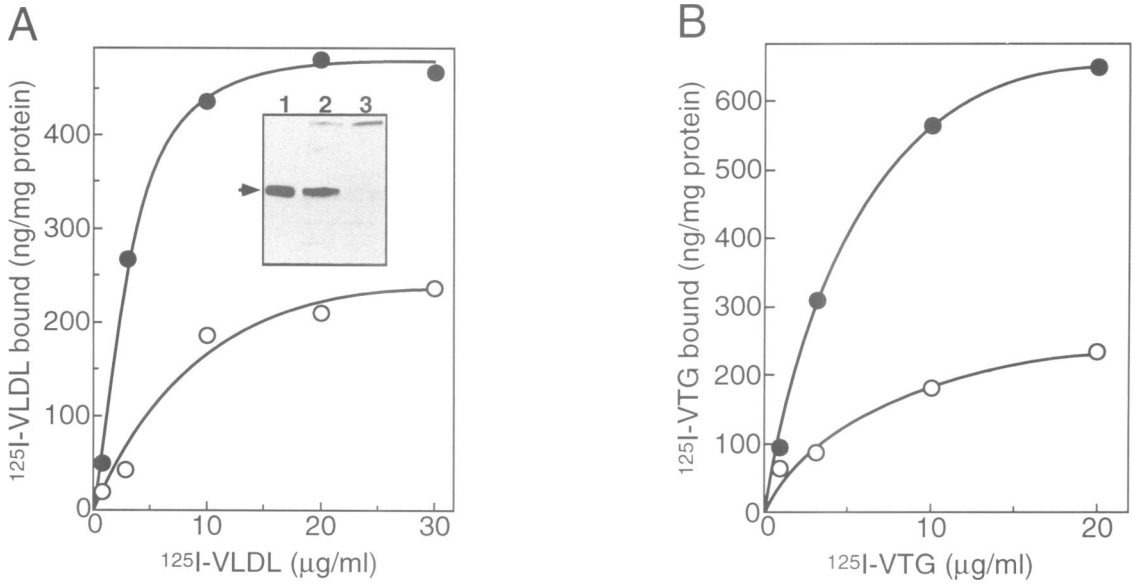


Fig. 3. Immunoblotting analysis of expressed VLDL/VTG receptor. COS-7 cells were transiently transfected with the VLDL/VTGR expression plasmid pCDMCVR-1 (lanes 2, 5 and 8; 70 μg protein/lane) or vector alone (lanes 3, 6 and 9; 70 μg cell protein/lane), and processed for immunoblotting following SDS–PAGE under non-reducing conditions as described in Materials and methods. Lanes 1 and 7 contained 5 μg, and lane 4 contained 1 μg of oocyte membrane protein. Immunoblotting incubations were performed with 2 μg/ml anti-VLDL/VTGR IgG (lanes 1–3), 20 μg/ml anti-C-terminal IgG (lanes 4–6), and 20 μg/ml non-immune IgG (lanes 7–9), respectively, followed by protein A-HRP and chemiluminescence detection. Exposure times were 5 min (lanes 1–3 and 7–9) and 2 min (lanes 4–6), respectively. The position of migration of the 95 kDa receptor is indicated by a closed circle.



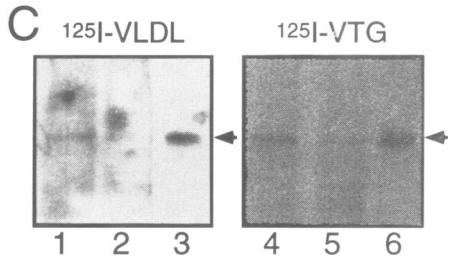


Fig. 4. Functional analysis of VLDL/VTG receptor expressed in COS-7 cells. Surface binding of (**A**) [^{125}I]VLDL (482 c.p.m./ng) and (**B**) [^{125}I]VTG (613 c.p.m./ng) to monolayers of pCDMCVR-1-transfected (closed circles) and vector-only transfected (open circles) COS-7 cells were determined as described in Materials and methods. The data are the average of duplicate determinations and represent high-affinity binding, which is the difference between binding in the absence and presence of excess unlabelled ligand (1 mg/ml VLDL, A; and 750 μg/ml VTG, B). In (**C**), an aliquot of the cells (60 μg protein/lane) used in (A) and (B) was subjected to ligand blotting with the same ^{125}I-labelled ligands at 4 μg/ml. Lanes 1 and 4, two dishes each of pCDMCVR-1 transfected cells; lanes 2 and 5, two dishes each of control cells; lanes 3 and 6, 0.15 μg of oocyte membrane protein. Autoradiography was for 30 h. The arrow indicates the position of the 95 kDa VLDL/VTGR. The insert in (A) shows the results of immunoblotting with the anti-C-terminal IgG performed as in Figure 3; lane 1, 1 μg oocyte membrane protein; lanes 2 and 3, 60 μg protein of pCDMCVR-1 transfected or control COS-7 cells, respectively.

Ligand binding function of the receptor expressed in COS-7 cells

In order to test whether the expressed protein is capable of binding both VLDL and VTG as demonstrated for the isolated oocyte receptor (Barber *et al.*, 1991), we performed surface ligand binding studies on the transfected COS-7 cells. As demonstrated in Figure 4, cells expressing the chicken VLDL/VTGR showed saturable, high affinity binding of both ligands, with maximal amounts of binding 2- to 3-fold higher than that of control-transfected cells. An exact determination of binding parameters for the expressed heterologous receptor is not possible due to saturable ligand binding to endogenous sites; however, maximum binding of VLDL and VTG to transfected cells were comparable, and the K_d values for both ligands were in the range of 3–5 μg/ml, both in excellent agreement with previous results on isolated oocyte receptor (Barber *et al.*, 1991).

In order to test whether the observed increased binding to the transfected COS-7 cells was indeed mediated by the 95 kDa chicken receptor, we performed ligand blotting experiments on detergent-solubilized pCDMCVR-1-transfected and control cells in parallel to the surface binding study. As shown in Figure 4C, in the transfected cells [^{125}I]VLDL (lane 1) and [^{125}I]VTG (lane 4) bound to a protein with the same electrophoretic mobility as the receptor in chicken oocyte membrane extracts (lanes 3 and 6). This experiment also revealed low levels of an endogenous 95 kDa COS-7 cell protein capable of binding both chicken ligands (lanes 2 and 5); presumably, this protein represents the simian VLDLR (cf. Webb *et al.*, 1994) and is responsible, at least in part, for the observed small amount of saturable ligand binding to control cells. The insert in Figure 4A shows the immunological identification of the expressed chicken receptor (cf. Figure 3). Thus, the combination of results of cell surface binding, ligand- and immunoblotting demonstrates that expression of the chicken cDNA in a heterologous cell system leads to bona fide VLDL/VTGR function of the 95 kDa protein.

Transmembrane orientation of the VLDL/VTG receptor

The unusually high concentration of VLDL/VTGR protein in the oocyte, and in particular, its presence in coated vesicles (Barber *et al.*, 1991; Stifani *et al.*, 1991; Shen *et al.*, 1993), allowed us to test directly for and/or confirm the putative orientation of the receptor within the plasma membrane. To this end, we isolated endocytic coated vesicles from oocytes (Nandi *et al.*, 1982; Shen *et al.*, 1993), subjected them to proteolytic treatment with pronase, and analysed the products by immunoblotting of the receptor protein with the antibodies directed against the

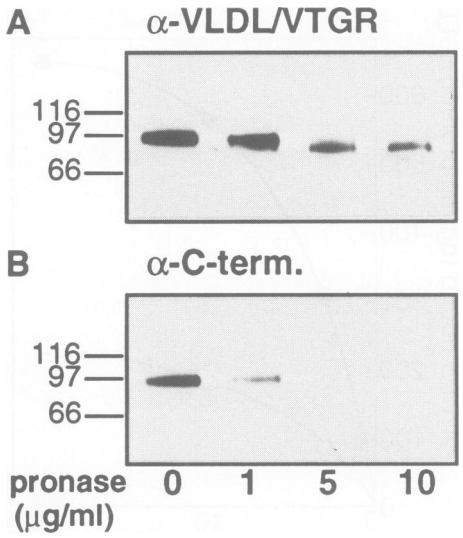


Fig. 5. Transmembrane orientation of the VLDL/VTG receptor in coated vesicles. The samples of coated vesicles (4.5 μg) were incubated with 0, 1, 5 or 10 μg/ml pronase for 1 h at 4°C. Digestion was terminated by addition of TCA. The precipitate was subjected to non-reducing electrophoresis on 4.5–18% SDS–polyacrylamide gradient gels and transferred to nitrocellulose. Western blotting was performed with polyclonal rabbit antibodies against (**A**) the 95 kDa protein (3 μg/ml) or (**B**) the C-terminal 14 residues of the VLDL/VTG receptor (1 μg/ml). Bound antibodies were detected by enhanced chemiluminescence. Exposure time was 6 min for (A) and 1 min for (B). Molecular masses in kDa are indicated.

receptor's C-terminus and the intact receptor, respectively (see Figure 3). As Figure 5 demonstrates, progressive proteolytic treatment abolished the C-terminal region of the receptor so that recognition by the anti-C-terminus IgG was lost (Figure 5B). The proteolysis-resistant portion of the receptor protein reacted with anti-VLDL/VTGR IgG, and became smaller by ~6 kDa (Figure 5A), the size of the C-terminal receptor domain adjacent to the proposed membrane spanning domain (54 amino acids, cf. Figure 1). This establishes, importantly, that the hitherto unknown transmembrane orientation of VLDLRs is identical to that of LDLRs, with the cluster of ligand binding repeats at the N-terminus exposed on the cell surface (Schneider *et al.*, 1983).

Tissue expression and sex chromosomal location of the VLDL/VTGR gene

The availability of VLDL/VTGR cDNA enabled us to analyse various chicken tissues for the expression of the gene by Northern blotting (Figure 6). As expected from the biochemical data, a transcript of the expected size (~3.5 kb) was highly abundant in the ovary. Much lower levels (0.5–1% of that in ovaries) of mRNAs of similar size were found in heart and muscle, the major sites of expression of mammalian VLDLR genes (Takahashi *et al.*, 1992; Gåfvels *et al.*, 1993; Webb *et al.*, 1994). Northern blotting under these conditions failed to detect message in brain, liver, adrenal gland, spleen, lung, kidney, small intestine and cultured embryo fibroblasts (not shown). However, RT-PCR using specific primers for amplification of the EGF precursor homology domain suggested very low levels of receptor mRNA in brain, kidney and liver (data not shown). Thus, molecular biological and biochemical criteria suggest that, in contrast to mammals,

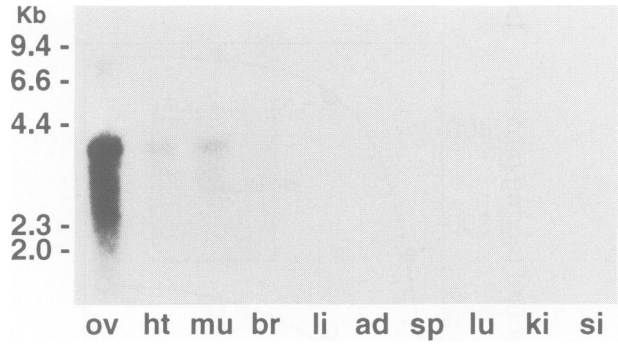


Fig. 6. Distribution of the VLDL/VTG receptor mRNA in chicken tissues. Poly(A)$^+$ RNA (2.5 μg) isolated from the chicken ovary (ov), heart (ht), muscle (mu), brain (br), liver (li), adrenal gland (ad), spleen (sp), lung (lu), kidney (ki) and small intestine (si) were denatured and separated by electrophoresis on a 1.0% agarose gel. Hybridization analysis was carried out using as probe a mixture of ^{32}P-labelled 1.2 kb *Xho*I–*Bgl*II and 0.7 kb *Bgl*II–*Bgl*II fragments excised from clone CVR-1 (see Materials and methods). Autoradiography was at −70°C for 2 days with an intensifying screen. λ DNA digested by *Hin*dIII was used as size marker (in kb).

the ovary is by far the most prevalent site of VLDLR expression in the avian species.

This finding, together with our previous results (Nimpf *et al.*, 1989) that pinpointed the absence of VLDL/VTGR function as the underlying defect in a mutant non-laying strain of chickens (termed 'restricted ovulator', R/O; Ho *et al.*, 1974), prompted us to attempt to identify the chromosome carrying the gene locus. Localization was simplified by the fact that breeding studies had established that the R/O phenotype [lack of growth of oocytes (R/O), non-laying, and severe hyperlipidaemia; Ho *et al.*, 1974] was due to a single gene defect carried on the sex chromosome Z (Jones *et al.*, 1975; McGibbon, 1977; note: normal male birds have two Z chromosomes; females are specified by one Z and one W chromosome). In order to confirm the localization of the VLDL/VTGR gene on the Z chromosome, Southern blot analysis was performed on genomic DNA obtained from normal roosters and hens using as probe the full length CVR-1 cDNA. Figure 7A shows the results obtained with *Hin*dIII-digested DNAs. There was no apparent difference between the DNA of male and female animals in the sizes of hybridizable restriction fragments; however, the signal of each fragment in male DNA was clearly stronger than that in an equal amount of female DNA. Quantification by densitometry of the signal intensities of each band revealed that males carry twice as many VLDL/VTGR gene copies as females (Figure 7B). This is in perfect agreement with our notion that the VLDL/VTGR gene locus is on the Z chromosome in the chicken, and is strongly supported by the R/O model (Ho *et al.*, 1974; Jones *et al.*, 1975; McGibbon, 1977). As a corollary to this finding, carrier roosters do not show an abnormal phenotype (Ho *et al.*, 1974; McGibbon, 1977), because the gene is expressed almost exclusively in ovaries (Figure 6).

Localization of VLDL/VTGR mRNA in oocytes

Previous ultrastructural immunocytochemistry (Barber *et al.*, 1991; Shen *et al.*, 1993) indicated that the 95 kDa protein is localized in the plasma membrane and periphery of rapidly growing oocytes (diameter >5 mm), but is

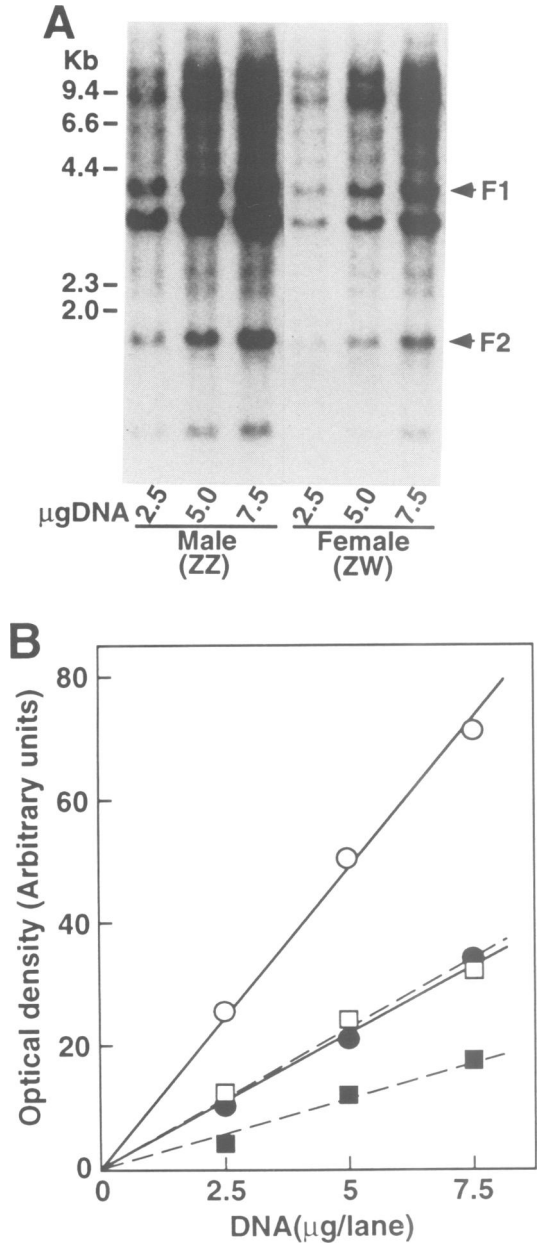


Fig. 7. Hybridization analysis for the localization of the VLDL/VTG receptor gene on sex chromosome Z. (**A**) Southern blot hybridization analysis of the VLDL/VTG receptor gene was performed. The indicated amounts of genomic DNA extracted from the blood cells of male and female chickens were digested with *Hin*dIII, and separated by electrophoresis on a 0.8% agarose gel. Hybridization analysis was carried out using full-length CVR-1 as probe. Autoradiography was at −70°C for 1 day with an intensifying screen. (**B**) The signal intensities of two of the hybridizing DNA fragments (F1 and F2, A) were quantified by densitometric analysis. The values for F1 and F2 are indicated by circles and squares, respectively (male: open, female: closed).

distributed throughout the cytoplasm in immature, quiescent oocytes <3 mm diameter. *In situ* hybridization with a VLDL/VTGR cDNA probe revealed that such apparent growth phase-dependent localization within oocytes is also displayed by the receptor transcript (Figure 8). In growing oocytes ~5 mm diameter (Figure 8, panel A, left upper corner), the mRNA is massively concentrated in a spherical zone underlying the plasma membrane, with fewer silver grains also present in the region that probably represents the zone of intercalation of oocyte surface microvilli and granulosa cell extensions (cf. Shen, *et al.*, 1993; Figure 8B). In contrast, the VLDL/VTGR mRNA is evenly distributed throughout the cytoplasm of oocytes that have not entered the rapid growth phase, such as the two shown in Figure 8 (panel A, right side). For control of both probe- and cell-type specificity of our procedure, we show that the mRNA specifying the somatic cell-specific LRP (Nimpf *et al.*, 1994) is indeed absent from oocytes as shown by mere background level staining, whereas it is present at very high levels in theca cells (Figure 8).

Discussion

The finding that the chicken oocyte 95 kDa receptor previously shown to be responsible for the uptake of VLDL and VTG into growing oocytes (George *et al.*, 1987; Nimpf *et al.*, 1989; Stifani *et al.*, 1990; Barber *et al.*, 1991; Shen *et al.*, 1993) is a homologue of the mammalian so-called VLDL receptor rather than the LDL receptor was somewhat surprising at first. The chicken LDL receptor, a 130 kDa sterol-regulated protein of somatic cells, does not bind apoE (Hayashi *et al.*, 1989), but the oocyte 95 kDa protein does recognize apoE, a hallmark property of LDLRs (Steyrer *èt al.*, 1990), and in addition, antibodies against mammalian LDLRs were crossreactive against the chicken oocyte receptor but not the chicken fibroblast receptor (George *et al.*, 1987; Hayashi *et al.*, 1989). We therefore anticipated that the oocyte lipoprotein receptor was a cell-specific type of LDLR. However, as reported here, this key receptor for oocyte growth is in fact related to the mammalian VLDLRs, warranting careful consideration of the possible physiological roles of VLDLRs in mammals and egg-laying species.

One of the remarkable aspects of VLDLRs is that the degree of conservation of their common domains, as indicated by amino acid identity, is greater than that among LDLRs. LDLRs of rabbit and man show identities of 75% (Yamamoto *et al.*, 1984, 1986), but rabbit and human VLDLRs are 97% identical; the identities between the eight-repeat receptors from chicken and rabbit as well as from chicken and man are 84%. This conservation, together with the fact that naturally occurring mutations in the VLDLR gene have not been reported to date, may indicate a vital importance of the receptors with eight ligand binding repeats. The present finding that the receptor critical to the most important biological function, i.e. reproduction, of the species *Gallus gallus* is such a protein, is in support of this hypothesis.

It is important to note that the oocyte-specific VLDL/VTGR of the chicken is the first eight-ligand-binding-repeat member of the LDLR family whose function is known. Previous kinetic and biochemical studies on the endocytosis of VTG in amphibian oocytes (Opresko and Wiley, 1987a,b; Wall and Patel, 1987) and ultrastructural investigations of VLDL uptake by chicken oocytes (Perry and Gilbert, 1979, 1985) strongly indicated a receptor-mediated mechanism for yolk precursor uptake. We have previously shown in biochemical (Barber *et al.*, 1991), cell biological (Shen *et al.*, 1993), and genetic studies

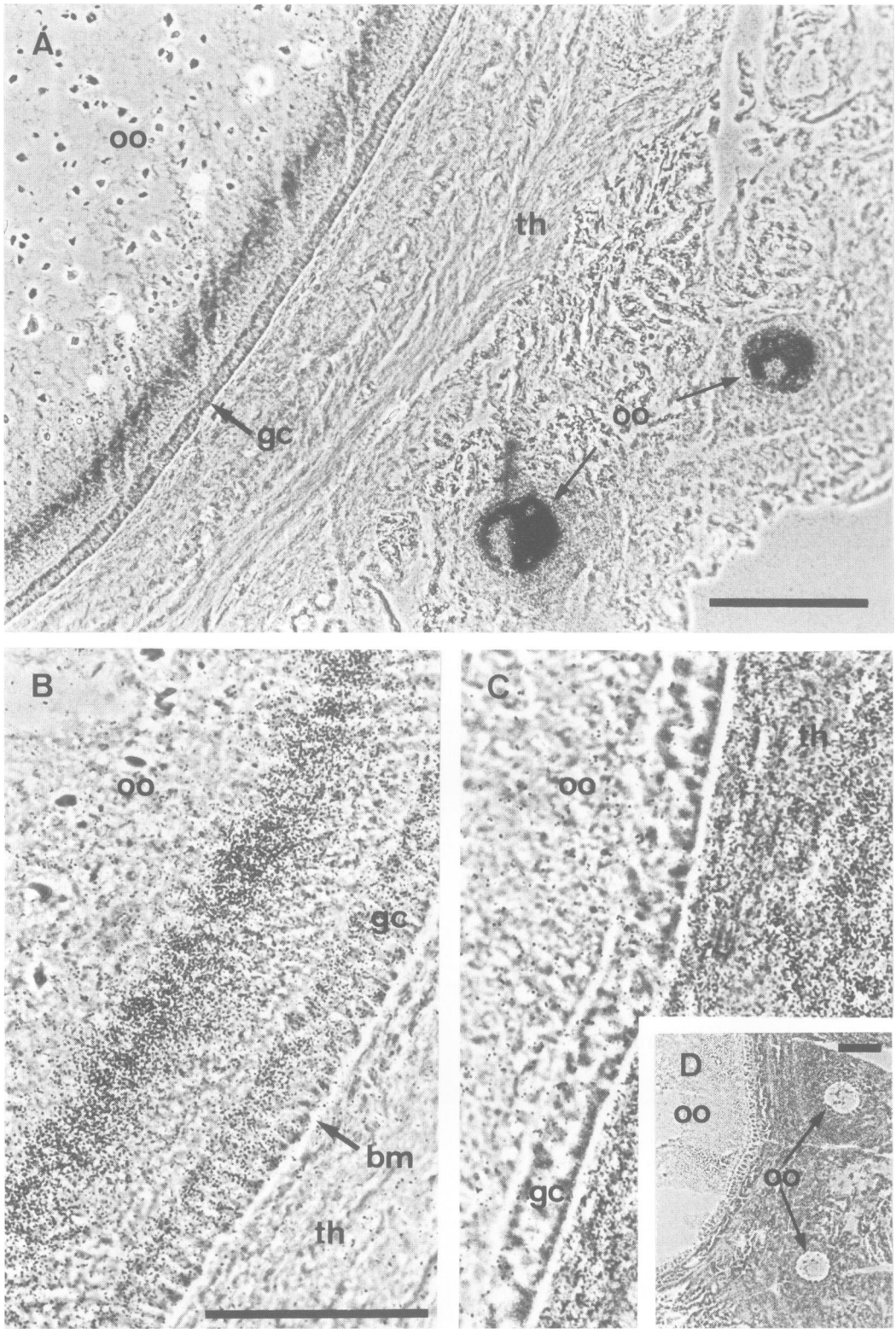


Fig. 8. *In situ* hybridization of LDLR family member transcripts in chicken ovary. Cryostat sections from ovaries were prepared and processed for *in situ* hybridization as described in Materials and methods. In (**A**) and (**B**), the cDNA probe was VLDL/VTGR-specific, and in (**C**) and (**D**), somatic-cell LRP-specific, respectively (see Materials and methods); exposure time was 21 days. The sections at lower magnification (A and D) contain an oocyte of ~5 mm diameter, surrounded by clearly discernible granulosa cells (gc) and theca interna (th), as well as two small previtellogenic oocytes (two each in A and D, 60–80 μm diameter). In (B), the pronounced basement membrane (bm) is also indicated. Bar: 200 μm (A) and 100 μm (B and D).

(Nimpf *et al.*, 1989) that the VLDL/VTGR is essential for yolk deposition and thus for oocyte growth and development. The receptor has high affinity for its physiological ligands, apoB of VLDL (Nimpf *et al.*, 1988) and the lipovitellin domain of VTG (Stifani *et al.*, 1990); it also recognizes apoE, an apolipoprotein that is implicated in the transport of intestinally derived lipoproteins in mammals, but which is not produced by birds (Steyrer *et al.*, 1990). Based on these ligand binding properties, we have previously suggested (Steyrer *et al.*, 1990) that the oocyte receptor is the product of an ancient gene that has retained the ability to interact with many, if not all, ligands of the present-day LDLR homologues. Thus, while evidence for recognition of apoE by mammalian VLDLRs is relatively scarce (Takahashi *et al.*, 1992; Sakai *et al.*, 1994), the properties of the chicken receptor support the notion that its mammalian homologue is involved in transport of apoE-containing lipoproteins. The role of mammalian VLDL receptors in delivering triglyceride to metabolically active tissues is under debate. In contrast, its role as importer of energy in the form of triglycerides into growing oocytes, stored in yolk for later use by the embryo, is established. Since the chicken receptor transports VTG, in addition to VLDL (Stifani *et al.*, 1990), this ligand has been previously suggested to represent a functional analogue of apoE (Steyrer *et al.*, 1990). Its biochemical properties, the presence of regions with sequence homology to apoE (Steyrer *et al.*, 1990), and, most of all, binding to lipoprotein receptors that recognize apoE [i.e. (V)LDLRs and LRP (Stifani *et al.*, 1991)], strongly support this notion.

The molecular basis for the interactions that result in binding of multiple ligands to single LDLR family members requires further structural refinement of the receptor molecules and their ligands. In comparing LDLRs with VLDLRs, the contribution of the eighth binding repeat, and also of the conserved acidic tetrapeptide Glu-Asp-Glu-Glu in the third repeat of VLDLRs, will be of particular interest. Studies to address these questions are now underway. These investigations may be feasible in the case of receptors containing single clusters of binding repeats, in contrast to the complex situation in LRP, which contains four clusters of between two and 11 binding repeats each (31–35 in total, depending on species). In such receptors, ligand specificity may be defined by the overall conformation of more than one cluster of repeats (Willnow *et al.*, 1994). In addition, the exact range of physiological ligands of LRP is not known. Current evidence suggests that LRP is a multifunctional receptor *in vivo*, responsible for the systemic clearance of spent, biologically inactive and/or unwanted plasmatic carrier complexes, as well as certain toxins (Bu *et al.*, 1992; Kounnas *et al.*, 1992; Nykjaer *et al.*, 1992; Orth *et al.*, 1992). Indeed, preliminary ligand binding experiments with the comparatively small chicken oocyte VLDL/VTGR suggest that it may be a superior model system to unravel structure/function relationships of LDLR gene family members.

The apparent relocalization of receptor mRNA during oocyte growth is another interesting finding of the current studies, as it is paralleled by observations at the protein level (Shen *et al.*, 1993). VLDL/VTGR mRNA is present in ovaries of immature hens (not shown), and the transcript is known to be located throughout the cytoplasm of non-vitellogenic (pre-growth) oocytes of laying hens. Presumably, at or before the onset of the rapid growth phase of vitellogenic oocytes in mature ovaries, the mRNA translocates to the desired site of receptor synthesis and localization, i.e. the peripheral region underneath the plasma membrane. Future studies will investigate whether (i) mRNA translocation is a general phenomenon in vitellogenic chicken oocytes, or a specific event for plasma membrane receptors mediating oocyte growth, such as the VLDL/VTGR , and (ii) the mRNA movement is triggered by endogenous (oocytic) or exogenous (somatic cell-derived) factors.

Materials and methods

Isolation of chicken VLDL/VTG receptor cDNA

Total RNA was extracted from adult chicken ovaries by the guanidium thiocyanate/CsCl method (Chirgwin *et al.*, 1979) and poly(A)$^+$ RNA was isolated using oligo(dT)-cellulose chromatography (Pharmacia). Single-stranded cDNA was synthesized from 1 μg of poly(A)$^+$ RNA using SuperScript reverse transcriptase (Gibco-BRL) and random primers. To obtain a chicken VLDL/VTG receptor cDNA fragment used as a probe for screening cDNA libraries, two degenerate oligonucleotides (A and B) were synthesized as PCR primers: A, 5′-AA(TC)AT(TC)TA-(TC)TGGAC(TCA)GA-3′ and B, 5′-GG(GA)TT(GA)TC(GA)AA(GA)-TTCAT-3′. The nucleotide sequences of primers A and B, respectively, were derived from the sequences of two tryptic peptides (I and III in Figure 1) obtained previously by microsequencing of the purified chicken VLDL/VTGR protein (Barber *et al.*, 1991). Amplification of cDNA was performed with the primers A and B using the GeneAmp PCR kit (Perkin-Elmer) on a Perkin-Elmer Thermal Cycler 480. PCR parameters were 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 30 cycles. Amplified products were separated by agarose gel electrophoresis, and products of ~800 bp were excised, purified using Geneclean II (BIO 101) and subcloned into the pGEM-T vector (Promega). Several clones were isolated and sequenced using Sequenase (US Biochemical). Clone C8-1, which encoded a sequence (839 bp open reading frame) highly homologous to VLDL and LDL receptors, was used as a probe for screening a chicken ovary cDNA library. This cDNA library was constructed from mRNA isolated from an ovary of a 6 month old White Leghorn hen according to the method of Okayama and Berg, as described previously (Kumabe *et al.*, 1992). In order to clone the full-length chicken VLDL/VTGR cDNA, ~3×10^5 recombinant phages were screened by plaque hybridization using ^{32}P-labelled (Megaprime DNA labelling kit, Amersham) C8-1 in a solution containing 5× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin), 1% SDS, 100 μg/ml salmon sperm DNA and 50% formamide at 42°C for 20 h. Subsequently, the filters were washed in 0.1× SSC, 0.1% SDS at 50°C. Several positive clones were obtained, and one of them, 1 CVR-1, was subcloned into pBluescript II KS (Stratagene) and sequenced on both strands. Nucleotide sequences were analysed by the GeneWorks computer program (IntelliGenetics Inc.).

Expression of chicken VLDL/VTG receptor in COS-7 cells

COS-7 cells (American Type Culture Collection) were seeded at a density of 1.5×10^6 per 80 cm^2 dish and incubated overnight in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 0.05 mM β-mercapto-ethanol (standard medium). CVR-1 in pBluescript was excised by *Xho*I and *Not*I, and subcloned into the cytomegalovirus promoter-driven expression vector pCDM8 (Invitrogen). The resulting plasmid, pCDMCVR-1 or pCDM8 (20 μg per dish), was transiently transfected into COS-7 cells by electroporation using a Bio-Rad Gene Pulser (Chu *et al.*, 1987). Dishes (60 mm diameter) were seeded with 4×10^5 cells each in standard medium; after 48 h, the cells were either prepared for immunoblotting, ligand blotting, or cell surface binding assays as described below.

Antibody production and immunoblotting

Antiserum against the C-terminus of the chicken VLDL/VTGR was prepared as follows. A synthetic peptide corresponding to the last 14

amino acids of the deduced amino acid sequence of the cloned cDNA for the chicken VLDL/VTGR was coupled to keyhole limpet hemocyanin (KLH) (Schneider *et al.*, 1983) and used for immunization of New Zealand White rabbits as described (Nimpf *et al.*, 1988). IgG fractions were purified from sera on columns of protein A–Sepharose CL-4B (Beisiegel *et al.*, 1981). Rabbit anti-VLDL/VTGR IgG was obtained by immunization with 95 kDa protein purified as described previously (Barber *et al.*, 1991); this IgG fraction crossreacts with oocyte-specific LRP (cf. Figures 3 and 4).

Transiently transfected COS-7 cells were washed three times with PBS and harvested in PBS containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and 2.5 μM leupeptin. Cells were pelleted by centrifugation and detergent extracts were prepared as follows. The cell pellet derived from one dish (80 cm^2) was resuspended in 75 μl of ice-cold solubilization buffer containing 200 mM Tris–maleate (pH, 6.5), 2 mM $CaCl_2$, 0.5 mM PMSF, 2.5 μM leupeptin and 1% Triton X-100 and kept on ice for 10 min. The extraction mixture was centrifuged at 300 000 *g* for 40 min at 4°C and the resulting supernatant was used for immunoblotting. Protein concentrations were determined by the method of Lowry *et al.* (1951).

One-dimensional gradient (4.5–18%) SDS–PAGE was performed according to Laemmli (1970) using a minigel system (Bio-Rad, Mini-Protean™ II Slab Cell). Samples were prepared in the absence of dithiothreitol (DTT) and without heating (non-reducing conditions). Electrophoresis was performed at 180 V for 60 min. Broad range M_r standards (Bio-Rad) were used. Electrophoretic transfer of the proteins to nitrocellulose membrane (Bio-Rad, pore size 0.45 μm) was performed in transfer buffer (26 mM Tris, 192 mM glycine) for 2 h at 200 mA, on ice, using the Bio-Rad Mini Transblot system. The transferred proteins were stained with 0.2% Ponceau S in 3% (w/v) TCA and destained with water. Western blotting was performed using specific rabbit antibodies at the concentrations indicated in the Figure legends, followed by protein A–horseradish peroxidase (HRP, Sigma) and the chemiluminescence detection method (ECL system, Amersham). Membranes were exposed for 0.1–5 min on Hyperfilm-ECL (Amersham).

Surface binding of VLDL and VTG to transfected COS-7 cells and ligand blotting

VLDL and VTG were radiolabelled with ^{125}I as described previously (Barber *et al.*, 1991) to a specific radioactivity of 482 c.p.m./ng and 613 c.p.m./ng, respectively. All assays were performed on ice. Monolayers of COS-7 cells transiently transfected with pCDMCVR-1, and control cells (transfected with pCDM8), were incubated for 3 h in standard medium containing 2 mg/ml bovine serum albumin and the concentrations of radioiodinated and unlabelled ligands indicated in the legend to Figure 4. The medium was then removed and the monolayers carefully washed to remove unbound ligand as described previously (Hayashi *et al.*, 1989). Cell-associated radioactivity was determined by liquid scintillation counting following solubilization of the cells in 1 ml of 0.1 N NaOH; cell protein was determined from an aliquot of the solution by the method of Lowry *et al.* (1951). Ligand blotting following SDS–PAGE and electrophoretic transfer as described above was performed as reported previously (Barber *et al.*, 1991).

Preparation of coated vesicles and pronase digestion

Coated vesicles were prepared according to Nandi *et al.* using a $^1H_2O/^2H_2O$–8 % sucrose gradient (Nandi *et al.*, 1982). White follicles (2–5 mm diameter) from three adult chicken ovaries were homogenized in 2 vol of MES-buffer (0.1 M MES, pH 6.5, 1.0 mM EGTA, 0.5 mM $MgCl_2$, 3.0 mM NaN_3, 1.0 mM PMSF, 5.0 μM leupeptin) with a polytron twice for 20 s. The homogenate was centrifuged at 5000 *g* for 5 min. The pellet was resuspended in 2 vol of MES-buffer and centrifuged at 5000 *g* for 5 min. The supernatants from the two centrifugations were combined and centrifuged at 100 000 *g* for 1 h. The resulting pellet was suspended in MES-buffer and centrifuged at 10 000 *g* for 10 min. The resulting pellet was resuspended in MES-buffer and the centrifugation was repeated. The supernatants from the two centrifugations were combined and centrifuged at 100 000 *g* for 1 h. The pellet was resuspended in 3 ml of MES-buffer, centrifuged at 10 000 *g* for 10 min, the pellet again resuspended in 3 ml of MES-buffer and centrifuged at 10 000 *g* for 10 min. The combined supernatants (6 ml) were loaded on the top of 6 ml 8% sucrose in ^2H_2O and centrifuged at 80 000 *g* for 2 h. The pellet, resuspended in 300 μl MES-buffer without PMSF and leupeptin, was centrifuged at 20 000 *g* for 10 min. The supernatant was recovered and stored at 4°C. The protein concentration of the resulting CV-preparation was 1.5 mg/ml. Three microlitres of the preparation were incubated with pronase in a total volume of 15 μl at 4°C for 1 h. The final pronase concentrations were 1, 5 and 10 μg/ml. After incubation, TCA was added to a final concentration of 20%. The resulting precipitates were dissolved in 2×non-reducing Laemmli sample buffer and used for the following gel electrophoresis and Western blot analysis as described in antibody production and immunoblotting.

Northern and Southern blot analysis

For Northern blotting, poly(A)$^+$ RNA (2.5 μg) prepared from various tissues of adult (>6 months old) female chickens was denatured using glyoxal-dimethylsulfoxide, separated by electrophoresis on a 1.0% agarose gel, and blotted onto Hybond C Extra membrane (Amersham) using standard methods (Sambrook *et al.*, 1989). The probe used was a mixture of a 1207 bp *Xho*I–*Bgl*II fragment and a 711 bp *Bgl*II–*Bgl*II fragment of CVR-1 in pBluescript II KS. The membrane was hybridized at 42°C in 50% formamide, 5×SSC, 5×Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate (pH 7.0), 100 μg/ml salmon sperm DNA and ^{32}P-labelled probe. Washing was performed in 0.1×SSC, 0.1% SDS at 50°C. For Southern blotting, total genomic DNA was prepared from chicken blood cells (Sambrook *et al.*, 1989). Restriction digests were separated by electrophoresis on a 0.8% agarose gel and blotted onto Hybond C Extra membrane. Hybridization and washing conditions were as described for Northern blot analysis except that a cDNA fragment containing the entire coding sequence of chicken VLDL/VTGR was used as a probe. Filters were exposed to Fuji RX film with intensifying screens. The signal intensities of hybridizing restriction fragments on Southern blots were quantitated by densitometric analysis (Molecular Dynamics).

In situ hybridization

Follicles from the ovaries of adult hens were dissected in ice-cold PBS, embedded in Tissue-Tek OCT compound (Miles) and immediately frozen with 2-methylbutane (Janssen Chimica) which had been precooled in liquid nitrogen. Cryostat sections at 10 μm thickness were prepared, transferred to glass slides treated with 2% 3-aminopropyltriethoxysilane (Fluka) and stored at −70°C in boxes containing silica gel. After drying, the sections were fixed with 4% paraformaldehyde in PBS for 1 h at 23°C and rinsed with PBS, saline and water. The sections were depurinated with 0.2 M HCl for 20 min, treated with 2×SSC at 70°C for 30 min, serially dehydrated in ethanol and dried. Prehybridization was carried out for 2 h at 43°C in a solution containing 50% formamide, 10% dextran sulfate, 1× salt buffer (0.3 M NaCl, 10 mM sodium phosphate, pH 6.8, 5 mM EDTA, 10 mM Tris–HCl, 0.02% polyvinylpyrolidon, 0.02% Ficoll and 0.02% bovine serum albumin), 0.5 mg/ml salmon sperm DNA and 100 mM DTT. Sections were hybridized overnight at 38°C in the same solution as used for the prehybridization without salmon sperm DNA, containing the ^{35}S-labelled probe (3×10^6 c.p.m. per slide). The probes used were a 1207 bp *Xho*I–*Bgl*II fragment of VLDLR/VTGR cDNA as described above or a 1.1 kb fragment of the chicken LRP cDNA (nucleotide position 4030–5133; Nimpf *et al.*, 1994). Slides were then washed twice for 10 min each in 2× SSC containing 10 mM DTT at room temperature, three times for 40 min in 50% formamide, 2× SSC with 10 mM DTT at 50°C, twice for 30 min in 2× SSC at 50°C and twice for 40 min in 0.2× SSC at 50°C. The slides were dehydrated in ethanol containing 0.3 M ammonium acetate, dried and dipped in LM-1 Photoemulsion (Amersham). Exposure times were 10–30 days. The sections were stained with toluidine blue and mounted in Aquamount (BDH, Poole).

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