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Molecular Cloning of the Gene Sequences of a Major Apoprotein in Avian Very Low Density Lipoproteins†

Lawrence Chan,* Achilles Dugaiczyk, and Anthony R. Means

ABSTRACT: ApoVLDL-II is a major apoprotein in avian very low density lipoproteins (VLDL). Its synthesis in the cockerel liver is markedly stimulated by estrogen treatment [Chan, L., Jackson, R. L., O'Malley, B. W., & Means, A. R. (1976) *J. Clin. Invest. 58*, 368–379]. We have partially purified apoVLDL-II mRNA from the liver of estrogen-treated cockerels by the following procedures: total nucleic acid extract, oligo(dT)–cellulose chromatography, Sepharose 4B chromatography, repeat oligo(dT)–cellulose chromatography, and sucrose gradient ultracentrifugation. A double-stranded complementary DNA (ds cDNA) was synthesized from the partially (25–30%) pure mRNA and inserted into the *Pst*I site of the plasmid pBR322. Amplification of the chimeric plasmids was accomplished by transformation in *Escherichia coli* RRI strain, and clones were screened by direct colony transfer and in situ hybridization by using the partially pure [^{32}P]-apoVLDL-II cDNA probe. The DNAs of positive clones were isolated and further studied by the hybrid-arrested cell-free translation technique. One clone, pVL10, which inhibited the translation of apoVLDL-II mRNA, was further characterized by DNA partial sequencing. It was found to contain nucleotides which code for amino acids 62–75 of apoVLDL-II. Hybridization of a nick-translated [^{32}P]pVL10 *Hha*I/*Hph*I fragment to the apoVLDL-II mRNA resulted in a $R_0t_{1/2}$ of 5.6×10^{-3} with >90% completion of hybridization. When a similar nick-translated probe was used, apoVLDL-II mRNA sequences were quantified in cockerel liver RNA before and 12 h after a single injection (2 mg) of diethylstilbestrol (DES). Hormone treatment resulted in a 12 000-fold increase in the concentration of apoVLDL-II specific sequences within 12 h after DES. In contrast, such sequences were not detected (up to a R_0t of 3×10^3) in RNA samples isolated from the breast muscles of these animals.

The estrogen-treated cockerel has been used as a model system for investigations into the mechanisms of steroid hormone action as well as the molecular aspects of lipoprotein synthesis. In this animal, estrogen administration markedly stimulates lipoprotein synthesis, involving mainly very low density lipoproteins (VLDL)[1] (Hillyard et al., 1956; Luskey et al., 1974; Chan et al., 1976, 1977). There are two major apoproteins in avian VLDL which account for over 90% of the proteins in VLDL. They have been designated apoVLDL-I and apoVLDL-II. The plasma levels of both proteins are markedly stimulated by estrogen. ApoVLDL-II has been purified to homogeneity, and its primary sequence has been determined (Chan et al., 1976a,b; Jackson et al., 1977). The mRNA for apoVLDL-II has been isolated and its translation product characterized. The latter was found to be larger than the plasma protein by 23 amino acids, containing a highly hydrophobic signal sequence at its amino terminus (Chan et al., 1978, 1980). Estrogen treatment was shown to induce a rapid accumulation of apoVLDL-II mRNA, as demonstrated by in vitro translation assays of avian hepatic mRNA (Chan et al., 1976a, 1978, 1979, 1980).

We now report the purification of the apoVLDL-II structural gene sequence by molecular cloning of the ds cDNA of a partially purified apoVLDL-II mRNA. Conventional techniques of RNA purification were used to prepare an apoVLDL-II-enriched mRNA. Purification of the structural sequences was completed by molecular cloning of the ds cDNA synthesized from the partially purified apoVLDL-II mRNA, using the plasmid pBR322 as vector and *E. coli* strain RRI as host. One of the clones isolated was shown by hybridization analysis, hybrid-arrested cell-free translation, and partial DNA sequencing to contain an inserted apoVLDL-II DNA sequence. Furthermore, using a radiolabeled nick-translated apoVLDL-II DNA probe, we have demonstrated that hepatic apoVLDL-II sequences are markedly stimulated by estrogen.

Materials and Methods

Materials. Four-week-old White Leghorn cockerels were purchased from Animal Specialties Co. Animals received daily injections of diethylstilbestrol (DES), 2.5 mg in sesame oil daily for 1 week, and were sacrificed 24 h after the last injection.

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[1] Abbreviations used: VLDL, very low density lipoproteins; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; oligo(dT), oligo(thymidylate); DES, diethylstilbestrol; HART, hybrid-arrested cell-free translation; ds cDNA, double-stranded complementary DNA.

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Creatinine phosphate and creatine phosphokinase were purchased from Sigma Chemical Co. Oligo(dT)–cellulose (T3) was purchased from Collaborative Research, Sepharose 4B was from Pharmacia Fine Chemicals, sucrose (ribonuclease-free) and ultra-pure guanidine hydrochloride were from Bethesda Research, and agarose (electrophoretic grade) was from Bio-Rad Laboratories. Sodium dodecyl sulfate ($NaDodSO_4$) was from Atomergic Chemetals Corp. Deoxyribonucleoside triphosphates were from P-L Biochemicals and [^{3}H]dCTP, [^{3}H]dTTP, [^{3}H]leucine, [α-^{32}P]dCTP, and [α-^{32}P]dTTP from Amersham Corp. Restriction endonucleases were purchased from Bethesda Research Laboratories or New England Biolabs.

Wheat germ was a gift from General Mills, Inc. Avian myeloblastosis virus reverse transcriptase was supplied by Dr. J. W. Beard, Life Sciences, Inc. Calf thymus terminal deoxynucleotidyltransferase was obtained from Dr. Winston Salzer, University of California, Los Angeles. DNA polymerase I was a product of Boehringer. All other chemicals were reagent grade and were purchased from Fisher Scientific Co.

Preparation of Total RNA. Total RNA was extracted from the liver of estrogen-treated cockerels by the method of Cox (1968) as modified by Deeley et al. (1977). Livers were homogenized in 20 volumes of 8 M guanidine hydrochloride, 20 mM sodium acetate, pH 5.0, and 1 mM dithiothreitol at −20 °C. The homogenate was centrifuged at 10000 rpm in a JA-14 rotor in a J21 Beckman centrifuge for 20 min and filtered through cheesecloth. The RNA was precipitated by addition of 0.55 volume of 95% ethanol at −20 °C for 30 min and collected by centrifugation, and the precipitate was redissolved in guanidine hydrochloride buffer. The precipitation was repeated 3 times. The RNA was then dissolved in 20 mM Na_2EDTA, pH 7.0, and extracted with chloroform/1-butanol (4:1 v/v), and the RNA was precipitated in two volumes of 95% ethanol at −20 °C overnight.

Oligo(deoxythymidylate)–Cellulose Chromatography. The total RNA was dissolved in 2 mM Na_2EDTA, heated at 70 °C for 2 min, quick-cooled in ice water, adjusted to 0.5 M KCl and 0.01 M Tris-HCl, pH 7.5, and subjected to oligo(dT)–cellulose chromatography by the method of Aviv & Leder (1972).

Sepharose 4B Chromatography and Repeat Oligo(dT)–Cellulose Chromatography. Chromatography of the poly(A)-enriched RNA on a Sepharose 4B column was performed as described previously (Woo et al., 1974), with the exception that the sample was first heated at 70 °C for 2 min and then quick-cooled in ice water to denature the RNA before application on the column. The apoVLDL-II mRNA activity in individual fractions was assayed in a wheat germ translation system. Fractions containing apoVLDL-II mRNA activity were combined and subjected to oligo(dT)–cellulose chromatography as described above.

Sucrose Gradient Centrifugation. mRNA recovered from the second oligo(dT)–cellulose chromatography step was heated at 70 °C for 2 min, quick-cooled, and applied to 12.4 mL of linear gradients of 13–25% sucrose in 40 mM Tris-HCl, pH 7.0, 1 mM sodium acetate, 2 mM Na_2EDTA, and 1% $NaDodSO_4$ at 39000 rpm for 16 h at 25 °C in a Beckman SW40 rotor. The gradients were fractionated into 0.5-mL samples, and the RNA was precipitated with ethanol, redissolved in water, and assayed for apoVLDL-II mRNA activity by the translation assay.

Translation Assay. RNA purification was monitored by translation in a heterologous cell-free protein-synthesizing system derived from wheat germ which was prepared by the method of Roberts & Paterson (1973) as described previously (Chan et al., 1976). [^{3}H]Leucine was used as the radioactive amino acid precursor. Total translational activity was determined by precipitation in 15% trichloracetic acid, following heating in the acid to 95 °C for 5 min. Specific apoVLDL-II mRNA activity was determined by immunoprecipitation by using monospecific antibody raised in goats against purified apoVLDL-II as described previously (Chan et al., 1976a, 1980). Immunoprecipitated products were analyzed by electrophoresis on 1.5 mm thick 12.5% polyacrylamide slab gels containing 0.1% $NaDodSO_4$ as described by Laemmli (1970), followed by fluorography (Bonner & Laskey, 1974) as described previously (Chan et al., 1980).

Enzymatic Synthesis of Double-Stranded cDNA. A cDNA copy of the apoVLDL-II mRNA was synthesized by using AMV reverse transcriptase and oligo(dT) primer. The reaction was carried out in 200 μL containing 50 mM Tris (pH 8.3), 50 mM KCl, 6 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1 mM each of dATP, dGTP, and dTTP, 0.5 mM [^{3}H]dCTP (10 μCi), 0.125 μg/mL oligo(dT)$_{12-18}$, 40 μg/mL actinomycin D, 15 units of the enzyme, and 50 μg of mRNA/mL at 46 °C for 30 min. The RNA–cDNA hybrid was separated from free nucleotides by Sephadex G-100 column chromatography. After alkaline hydrolysis of RNA (0.1 N NaOH, 30 °C, 5 h), the cDNA was converted to ds cDNA by the same reverse transcriptase by using the same reaction conditions as described above for synthesis of the first strand, except that KCl and actinomycin D were omitted and the reaction time was extended to 2 h.

S_1 Nuclease Treatment of ds cDNA. One microgram of ds cDNA was incubated with 30 units of S_1 nuclease at 37 °C for 1 h in 100 μL of a buffer containing 0.03 M sodium acetate (pH 4.5), 0.3 M NaCl, and 4 mM $ZnCl_2$ (Vogt, 1973). The sample was extracted with phenol, followed by ether, and then precipitated with 2 volumes of 95% ethanol at −20 °C.

Construction of Chimeric Plasmids. Poly(dC) residues were added to the 3′ termini of S_1-treated ds cDNA by terminal deoxynuclotidyltransferase (Bollum, 1974). The final reaction volume of 50 μL contained 100 mM potassium cacodylate (pH 7.0), 4 mM β-mercaptoethanol, 1 mM $CoCl_2$, 100 μM dCTP, 8–10-pmol termini of ds cDNA, and 10 units of enzyme. An average of $(dC)_{20}$ was polymerized per 3′ terminus. The tailed DNA was annealed with pBR322 DNA that had been linearized by *Pst*I and tailed with dG_{15} by the same terminal transferase. These plasmids were used to transform (Mandel & Higa, 1970) *E. coli* strain RRI.

Biosafety Precautions. All bacterial transformations were carried out according to the National Institutes of Health Guidelines (*Fed. Regist.*, 1976). Transformation and the initial steps of plasmid purification were performed under approved P-3 physical containment. All bacterial transfers were carried out by using approved vectors and hosts and were performed in a certified laminar flow hood in the P-3 containment facility.

Transformation of E. coli RRI. Aliquots of an overnight culture of *E. coli* RRI (0.5 mL) were inoculated into 50-mL L-broth. The bacterial culture was grown at 37 °C to A_{600} = 0.6 and collected by centrifugation at 4 °C. Cells were washed at 4 °C with an equal volume of 100 mM NaCl and 25 mM Tris (pH 7.4), resuspended in 0.5 volume of 50 mM $CaCl_2$, 100 mM NaCl, and 25 mM Tris (pH 7.4), and chilled on ice for 30 min. Cells were collected by centrifugation and resuspended in 0.1 original volume in the same $CaCl_2$ containing buffer. Cells (0.2 mL) were added to 50 μL of the annealed chimeric plasmid DNA mixture. After 40 min at 0 °C and 90 s at 40 °C, the transformation mixture was

diluted to 5 mL with L-broth and incubated at 37 °C for 40 min. Aliquots (50 μL) were plated on L-agar plates containing 15 mg/L tetracycline.

Direct Colony Transfer and in Situ Hybridization. Tc^R colonies were directly transferred to nitrocellulose filter disks. A filter was placed over the surface of the agar plate and allowed to soak thoroughly. It was then carefully removed to avoid smearing of the colonies. All colonies on the plate were transferred onto the filter in this procedure, and the residual cells were allowed to grow into colonies at 37 °C. This plate then served as a masterplate. The plate was returned to the 37 °C incubator for 48 h. The colonies on the replica filters were lysed in situ by the method of Grunstein & Hogness (1975). The dried filters containing denatured DNA were treated with 0.02% ficoll, 0.02% poly(vinylpyrrolidone), and 0.02% bovine serum albumin (Denhardt, 1966; Botchan et al., 1976) at 68 °C. Washing and hybridization with [^{32}P]cDNA synthesized from partially purified apoVLDL-II mRNA were performed as described by Botchan et al. (1976). Clones containing a complementary ds cDNA insert were identified by autoradiography, and the positive colonies on the master plate was streaked onto fresh Tc plates for further analysis.

Isolation of Recombinant Plasmid DNA. Recombinant plasmid DNA was prepared by amplification of pBR322-ds cDNA recombinants in RRI. Cells were grown in M9 medium at 37 °C to an optical density of 0.8 at 600 nm. Chloramphenicol was added to 20 mg/L, and the culture was incubated for an additional 12 h. Cleared lysates were prepared from these cells by the method of Katz et al. (1973) and chromatographed through a Sephadex G-100 column. Recombinant plasmid DNA was then purified by the method of Katz et al. (1977).

Slab Gel Electrophoresis. Digestion products of plasmid DNA were separated by electrophoresis on 2% agarose slab gels in 50 mM Tris (pH 8.4), 20 mM sodium acetate, 18 mM sodium chloride, and 2 mM EDTA (Helling et al., 1974). Electrophoresis was performed in the same buffer for 4 h at 100 V. The gel electrophoresis apparatus was made by E-C Apparatus (St. Petersburg, FL). DNA was visualized with ultraviolet light after staining in 5 μg/mL ethidium bromide or by radioautography when [^{32}P]nucleotide was used to label the DNA. DNA species were eluted from the gels by the procedure of Sharp et al. (1974). Electrophoresis on polyacrylamide slab gels was performed in a Bio-Rad slab gel apparatus. Polyacrylamide gels were cast from a solution that contained 5.68 g of acrylamide, 0.32 g of *N,N'*-methylenebis(acrylamide), 60 μL of *N,N,N,N'*-tetramethylethylenediamine (Temed), 0.6 mL of 10% ammonium persulfate in 50 mM Tris–borate, pH 8.3, and 1 mM EDTA per 100 mL.

Restriction Endonuclease Cleavage of Cloned ds cDNA. Reaction conditions for restriction endonuclease cleavage of cloned ds cDNA varied with the particular enzyme and were essentially as suggested by the supplier. Enzymes were added at units per microgram of DNA substrate and incubated 3 h at 37 °C. The reactions were stopped by addition of EDTA to 10 mM and heating at 68 °C for 5 min. Fragments of DNA generated were analyzed by 2% agarose or 6% acrylamide slab gel electrophoresis as described in a previous section.

Hybrid-Arrested Cell-Free Translation. DNA clones containing the structural gene for apoVLDL-II were identified by the hybrid-arrested cell-free translation (HART) method of Paterson et al. (1977). Recombinant plasmid DNA samples were linearized by the restriction enzyme *Eco*RI, phenol extracted, and subjected to HART. RNA samples used for hybridization consisted of apoVLDL-II mRNA purified through the first oligo(dT)–cellulose step. Hybridization conditions were those of Paterson et al. (1977). Control samples consisted of DNA–RNA mixtures which were directly translated without going through the 2-h 48 °C hybridization step. For translation, the nuclease-treated rabbit reticulocyte lysate system of Pelham & Jackson (1976) was used. [^{35}S]-Methionine (10–50 μCi) was the labeled amino acid precursor. Products of translation were analyzed on 15% polyacrylamide slab gels containing 0.1% $NaDodSO_4$ and 0.5 M urea (Chan et al., 1980). Gels were subjected to fluorography as described by Bonner & Laskey (1974).

DNA Sequencing. Sequencing of end-labeled DNA fragments was accomplished by the method of Maxam & Gilbert (1977).

Nick Translation. Cloned DNA fragments were labeled by nick translation by the procedure of Mackey et al. (1977), as modified by Roop et al. (1978). The reaction was performed in a final volume of 20 μL containing 50 M Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 90.12 mM dATP and dGTP, 150 μCi each of [^{32}P]dCTP (250 Ci/mmol) and [^{32}P]dTTP (250 Ci/mmol), 1.25 μM unlabeled dCTP and dTTP, and 100 ng of DNA. These compounds were assembled on ice, and 0.5 ng of DNase (Worthington, DPFF) was added. The mixture was incubated at room temperature for 60 s and immediately cooled in an ice-water bath. *E. coli* DNA polymerase I (4 μL, 16 units) was added and the mixture incubated at 14 °C. After 6 h, the reaction was stopped by the addition of 10 μL of 0.1 M EDTA, pH 7.0, and 100 μg of *E. coli* DNA and heating at 68 °C for 10 min. Unincorporated dNTPs were separated from the labeled DNA by gel filtration on a Sephadex G-100 column in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM NaCl. The radioactive fractions eluting in the void volume were pooled, adjusted to 0.2 M NaCl, and precipitated by the addition of 2 volumes of ethanol. The specific activity of the final product was 8×10^8 cpm/μg.

RNA Excess Hybridization against the Nick-Translated DNA Probe. The RNA excess hybridization experiments were performed in a final volume of 25–200 μL under conditions described by Harris et al. (1975), except that 5 μg/mL poly(C) was included in each vial to prevent reannealing of the poly(dC) to the poly(dG) in the two strands of the probe. RNA (12 ng/mL–5 mg/mL) was boiled together with 650 cpm of [^{32}P]DNA probe for 5 min. The mixture was incubated at 68 °C for time intervals ranging from 6 min to 24 h. After hybridization, the samples were treated with S_1 nuclease (1600 units), and the S_1 nuclease resistant hybrids were determined as previously described (Harris et al., 1975). Control incubations included vials with no cockerel liver RNA added or carrier RNA without addition of S_1 nuclease vials.

Results

Partial Purification and Translation Analysis of ApoVLDL-II mRNA. In the estrogen-treated cockerel, apoVLDL-II mRNA constituted about 15% of the total mRNA activity as determined by translation. When a number of techniques including total RNA extraction, oligo(dT)–cellulose chromatography, Sepharose 4B chromatography, repeat oligo(dT)–cellulose chromatography, and sucrose gradient ultracentrifugation on a 13–25% gradient were used, a mRNA preparation was obtained which consisted of about 30% apoVLDL-II mRNA by translation. The final RNA preparation yields a single band upon analysis on 3% acid urea agarose gels [by the method of Rosen et al. (1975)]. The RNA migrates as a 9S molecule and sediments under a single peak on sucrose gradients with an *s* value identical with that

Table I: Purification of ApoVLDL-II mRNA

	sp act.[a] (cpm/μg)	purifcn[b] (x-fold)	(apoVLDL-II synthesized)/(total protein synthesized) × 100[c]
total extract	1600	1	17.2
dT–cellulose bound	24000	15	20.0
Sepharose 4B peak	29500	18	22.1
dT–cellulose bound	47125	30	21.9
sucrose gradient peak	57240[d]	36[d]	26.6[d]

[a] Defined as the total amount of apoVLDL-II peptides synthesized in the wheat germ translation assay in response to 1 μg of the various RNA preparations. [b] Purification of apoVLDL-II mRNA over the total RNA in the preparation. [c] Determined as the ratio of immunoprecipitated apoVLDL-II in the wheat germ translation assay to the total Cl_3CCOOH-precipitated peptides. [d] The mRNA peak from the sucrose gradient contained small amounts of $NaDodSO_4$ which partially inhibited the translation assay. This inhibition makes accurate calculation of apoVLDL-II mRNA purity impossible, and the purification is probably a minimal number; the % apoVLDL-II synthesized over Cl_3CCOOH-precipitable peptides synthesized was also a minimal number since the antibody–antigen binding was also partially inhibited by the $NaDodSO_4$.

of rabbit globin mRNA (data not shown). However, since translation experiments reveal that the mRNA is only ~30% pure, the preparation must contain RNA species of very similar size not coding for apoVLDL-II. Table I is a summary of the purification and yield of apoVLDL-II mRNA as monitored by translation assays, using the wheat germ cell-free system.

Preparation and Amplification of Chimeric Plasmids. Since it was clear that the mRNA was not a homogeneous species and conventional methods of RNA purification were exhausted, molecular cloning techniques were employed to isolate and purify the coding apoVLDL-II DNA sequence. Double-stranded cDNA was prepared from the partially purified apoVLDL-II mRNA and the hairpin loop cut with S_1 nuclease as described under Materials and Methods. The predominant ds cDNA species contained 500–750 base pairs as estimated by agarose gel electrophoresis and was used for molecular cloning without further purification. The ds cDNA was labeled with [^{32}P]dCTP to an average length of 14 dCs per 3′ terminus by deoxynucleotidyltransferase. Chimeric plasmids were formed by annealing this [^{32}P]ds cDNA to *Pst*I-cut dG "tailed" pBr322. Ligation of the hybrid molecules as well as repair of any gaps which might occur due to the difference in length of the poly(dC) and poly(dG) regions occurred in vivo after transformation (Chang & Cohen, 1977). Transformation of RRI was performed as described under Materials and Methods by mixing the chimeric plasmids with $CaCl_2$-treated recipient cells. In this study, about 25% of the Tc^R clones contained inserted DNA complementary to [^{32}P]cDNA synthesized from partially purified apoVLDL-II. Plasmid DNA was prepared from positive clones as described under Materials and Methods.

To identify recombinant DNA clones containing apoVLDL-II structural sequences, the cloned cDNAs were subjected to HART. Ten clones were screened according to this technique, of which five were positive. Figure 1 is the fluorograph of a slab gel of HART products containing DNA from clones pVL_7, pVL_8, and pVL_{10}. Both pVL_8 and pVL_{10} caused inhibition of apoVLDL-II translation as evidenced by the partial disappearance of the preapoVLDL-II band on the slab gel, although inhibition was never complete. The incomplete inhibition may be related to the fact that neither pVL_8 nor pVL_{10} contained the complete apoVLDL-II sequence. The results of one negative clone, pVL_7, is also shown in Figure 1.

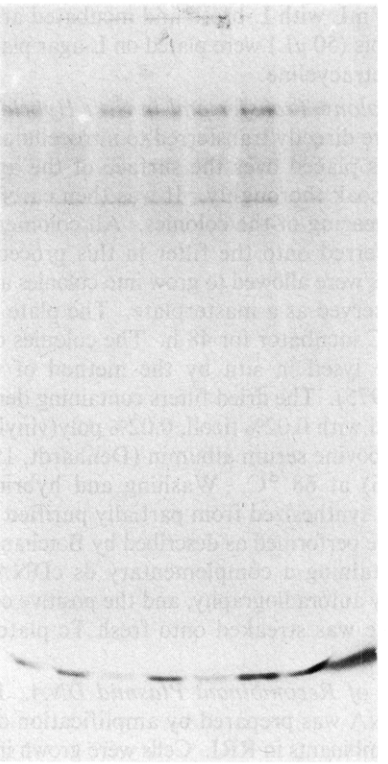

FIGURE 1: Hybrid-arrested cell-free translation (HART) of recombinant DNAs. HART was performed according to the method of Paterson et al. (1977). DNAs from recombinant clones were digested with *Eco*RI. To each HART assay tube was added 1 μg of DNA and 0.5 μg of cockerel liver RNA purified through the first oligo(dT)–cellulose step. Hybridization was at 48 °C for 45 min. Controls consisted of DNA added to the translation but not the hybridization phase of HART. Reaction products were analyzed by 15% acrylamide slab gels containing 0.1% $NaDodSO_4$ and 0.5 M urea. Lane 1, control for 2; lane 2, HART for pVL7 DNA; lane 3, HART for pVL_8 DNA; lane 4, control for 3; lane 5, HART for pVL_{10} DNA; lane 6, control for 5; lane 7, translation products of 0.2 μg of cockerel liver RNA; lane 8, immunoprecipitable apoVLDL-II synthesized in vitro.

Restriction Mapping of Recombinant Plasmid pVL_{10}: Confirmation of an ApoVLDL-II Insert by DNA Sequence. Since the complete amino acid sequence of apoVLDL-II is known, determination of the nucleotide sequence of the DNA insert would positively identify the presence of apoVLDL-II gene sequences. Recombinant plasmid pVL10 contained the longest cDNA insert of any of the clones. This insert was purified from most of the plasmid sequences by *Hha*I digestion and separation on a 2% agarose gel. An *Hha*I digest of clone pVL_{10} generated a DNA fragment 620 base pairs (bp) in size in addition to numerous smaller fragments that are also obtained from *Hha*I digestion of pBR322 DNA. The 620-bp DNA fragment was recovered from an agarose gel after electrophoretic separation from the smaller fragments. Restriction sites within this fragment were determined after single and multiple enzyme digests, and a partial restriction map is presented in Figure 2. The single *Hinf*I site within this fragment was labeled with [α^{32}P]dATP and reverse transcriptase. The resulting two DNA fragments were separated by acrylamide gel electrophoresis and sequenced by the procedure of Maxam & Gilbert (1977). The DNA sequence from the *Hinf*I site toward the adjacent *Pvu*II site is shown in Figure 3 and Chart I. This sequence corresponds to amino acids 62–75 of apoVLDL-II (Jackson et al., 1976). It confirms that

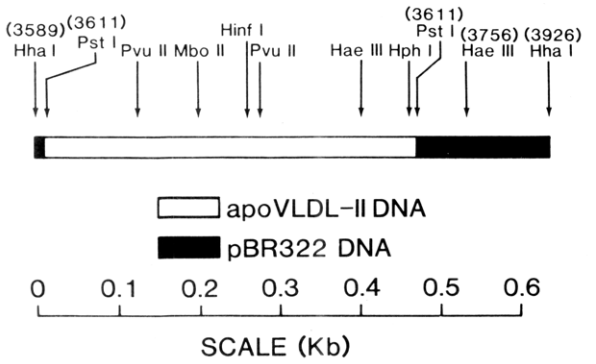

FIGURE 2: Restriction map of apoVLDL-II DNA in pVL_{10}. Restriction sites were determined after single and multiple enzyme digests. Black boxes represent plasmid DNA; numbers of parentheses are taken from Sutcliffe (1978) and represent restriction sites within pBR322 DNA. The open box represents apoVLDL-II insert DNA. The mRNA sequence is represented by the upper strand, the 3′ terminus being at the extreme right of the figure.

Chart I: Partial DNA Sequence of pVL_{10}[a]

Restriction Site	Hinf I								Pvu II
DNA	AGA	C/T TC	ACT	AAA	CTG	GCG	GAA	CAG	CTG
Amino Acid	Arg 60	Leu	Thr	Lys	Leu	Ala 65	Glu	Gln	Leu

ATG	GAA	AAA	ATA	AAG	AAC	CTT
Met	Glu 70	Lys	Ile	Lys	Asn	Leu 75

[a] The sequence was determined by the method of Maxam & Gilbert (1977) and is taken from data in Figure 3. Note that the sequence in this diagram is taken from the complementary sequence of Figure 3. Sequence was read from the *Hinf*I site toward 3′ terminus. From the sequence gel, the first codon which was definitely identified was ACT, corresponding to amino acid 62 or *Thr*. *Hinf*I site is reconstructed from the codons for *Arg*/*Leu*, and a *Pvu*II site is evident between the codons for *Gln* and *Leu*, 22 bases from the *Hinf*I site.

the inserted DNA of pVL_{10} contained apoVLD-II gene sequences.

Hybridization of [^{32}P]pVL_{10} *Probe to ApoVLDL-II mRNA.* Since double digestion of pVL_{10} with *Hha*I and *Hph*I resulted in a fragment which contained most of the inserted apoVLDL-II sequences with only 22 bases from the plasmid sequences, a nick-translated probe of this fragment was prepared by using [^{32}P]dCTP and [^{32}P]dTTP as precursors. The kinetics of hybridization of excess apoVLDL-II mRNA to this [^{32}P]pVL_{10} DNA probe are shown in Figure 4. It is apparent that it is a pseudo-first-order reaction achieving >90% completion (i.e., >45% of the 50% expected hybridization). The $R_0t_{1/2}$ value for the RNA–DNA hybridization was 5.6×10^{-3}. This compares to a $R_0t_{1/2}$ of 4.6×10^{-3} mol s L^{-1} for purified ovalbumin cDNA hybridization to its cDNA (Monahan et al., 1976). With the assumption that the complexity of apoVLDL-II mRNA is 750 and the complexity of ovalbumin mRNA is 1850, the expected R_0t for apoVLDL-II would be $\sim 2.0 \times 10^{-3}$ mol s L^{-1}, since

$$\frac{R_0t_{1/2} \text{ of apoVLDL-II mRNA}}{R_0t_{1/2} \text{ of ovalbumin mRNA}} = \frac{\text{complexity of apoVLDL-II mRNA}}{\text{complexity of ovalbumin RNA}}$$

The purity of the apoVLDL-II mRNA is approximately 36%. This is similar to the value of approximately 27% as estimated by translation analysis.

Stimulation of ApoVLDL-II mRNA Accumulation by Estrogen. We have previously demonstrated that estrogen

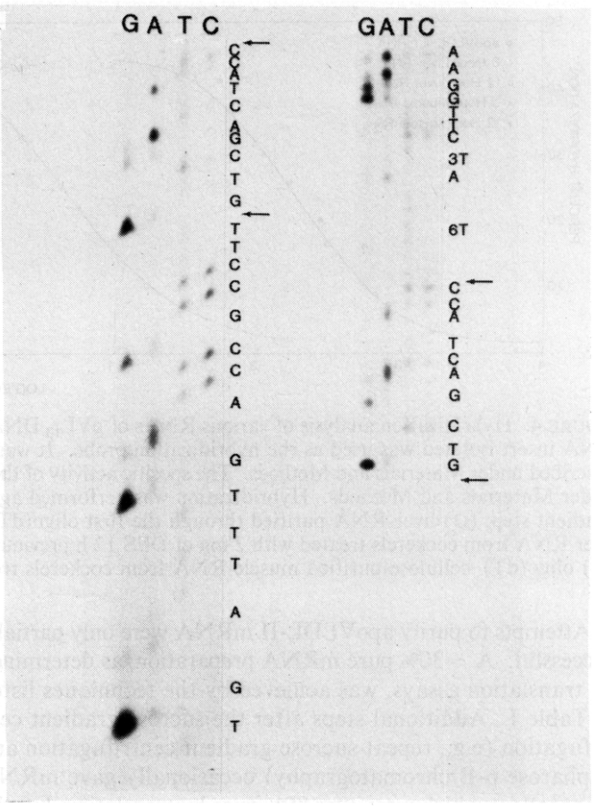

FIGURE 3: Radioautography of DNA sequence gels. End-labeled *Hinf*I fragment of pVL_{10} was sequenced toward the adjacent *Pvu*II site and analyzed on 20% (left) and 8% (right) acrylamide gels. The overlapping sequence on the two gels is enclosed by the two arrow heads.

treatment in the cockerel results in an accumulation of intrahepatic mRNA for apoVLDL-II as measured by translation assays (Chan et al., 1976a,b, 1980). The following experiment was performed to examine whether a comparable increase in apoVLDL-II specific RNA sequences is also detected by hybridization to the pVL_{10} probe. Four-week-old cockerels were treated with a single injection of DES (2-mg pellet, subcutaneously). Total RNA was isolated from the livers of untreated controls and from those livers obtained from animals 12 h after DES treatment. As control, RNA was also isolated from the breast muscles at 0 and 12 h after DES. Poly(A)-enriched RNA was prepared from each sample by oligo(dT)–cellulose chromatography. Hybridization was then carried out against the [^{32}P]pVL_{10} DNA probe. Figure 4 shows the results of such analyses. In the absence of estrogen, the $R_0t_{1/2}$ value of hybridization was 2.8×10^3. Twelve hours after the DES treatment, it decreased to 2.3×10^{-1}. This represents a 12 000-fold increase in the concentration of apoVLDL-II specific sequences in 12 h and documents that we have cloned an estrogen-responsive gene. In contrast, there were no detectable apoVLDL-II mRNA sequences in the mRNA isolated from breast muscles either before or after estrogen treatment.

Discussion

Lipoproteins are a complex class of proteins characterized by their ability to bind and transport lipids in plasma (Jackson et al., 1976). While there is considerable information on the physicochemical characteristics of these proteins, relatively little is known of their mode of biosynthesis. In the past 5 years, we have utilized a major apoprotein, apoVLDL-II, in VLDL in the estrogen-treated cockerel, as a model for the study of the regulation of apolipoprotein biosynthesis (Chan et al., 1979).

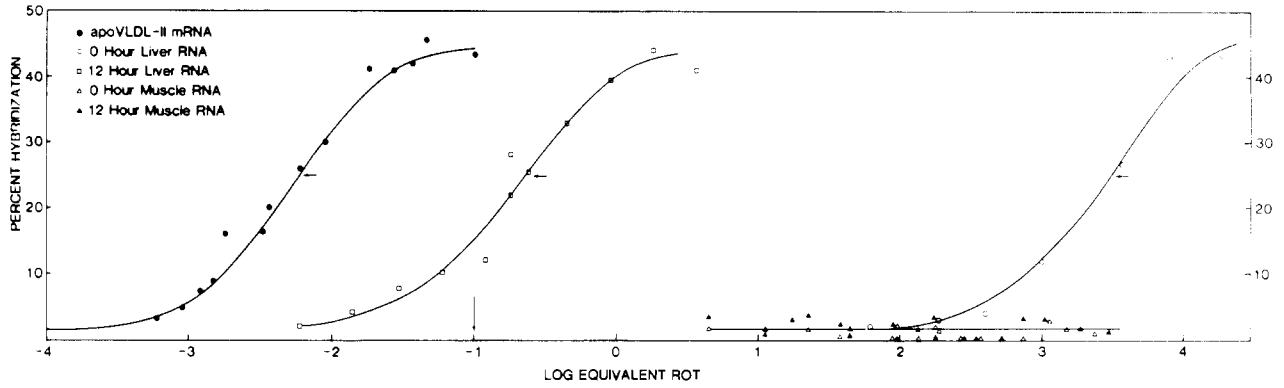

FIGURE 4: Hybridization analysis of various RNAs of pVL_{10} DNA. The *Hha*I fragment of pVL_{10} was recut with *Hph*I, and the apoVLDL-II DNA insert isolated was used as the hybridization probe. It was nick-translated, using [^{32}P]dCTP and [^{32}P]dTTP as labeled precursors, as described under Materials and Methods. The specific activity of the [^{32}P]DNA was 8×10^8 cpm/μg. Hybridization conditions were as described under Materials and Methods. Hybridization was performed against excess RNA. (●) ApoVLDL-II mRNA purified through the sucrose gradient step; (○) liver RNA purified through the first oligo(dT)–cellulose step from untreated cockerels; (□) oligo(dT)–cellulose-purified liver RNA from cockerels treated with 2 mg of DES 12 h previously; (△) oligo(dT)–cellulose-purified muscle RNA from untreated cockerels; (▲) oligo(dT)–cellulose-purified muscle RNA from cockerels treated with 2 mg of DES 12 h previously.

Attempts to purify apoVLDL-II mRNA were only partially successful. A ~30% pure mRNA preparation, as determined by translation assays, was achieved by the techniques listed in Table I. Additional steps after the sucrose gradient centrifugation (e.g., repeat sucrose gradient centrifugation and Sepharose 6-B chromatography) occasionally gave mRNA preparations which were over 80% pure by translation, but the yield was generally very poor. Since large amounts of the nucleic acid sequence were needed for subsequent studies on the structure and expression of the apoVLDL-II gene, we decided to clone the ds cDNA of a partially (~30%) purified RNA preparation. This strategy proved productive in that DNAs containing apoVLDL-II sequences were readily identified in the recombinant plasmid DNA clones.

Double-stranded apoVLDL-II cDNA was cloned by using the plasmid pBR322 and the host RRI. Preliminary identification of apoVLDL-II sequences in the clones was obtained by the hybrid-arrested cell-free translation assay of Paterson et al. (1977). While there was definite inhibition of the preapoVLDL-II band by pVL_{10}, the inhibition was incomplete. Therefore, we proceeded to confirm the authenticity of the cloned sequence by DNA sequencing. A perfect correspondence was found between the DNA sequence of a restriction fragment from a cloned DNA insert and a stretch of 14 amino acids in apoVLDL-II. These data provided further evidence that we had amplified at least a portion of the apoVLDL-II structural gene. On the other hand, since we have not completed the nucleotide sequence corresponding to the entire amino acid sequence of apoVLDL-II, it is quite possible that we have cloned the DNA sequence of an apoVLDL-II-related protein having extensive amino acid homology to the protein originally sequenced by Jackson et al. (1977). For simplicity, however, this cloned DNA sequence will be referred to as "apoVLDL-II DNA" clone. Additional studies with restriction enzyme mapping of pVL_{10} revealed that the plasmid contained ~500 bases of the apoVLDL-II mRNA sequence. It can be estimated that the cloned fragment contains sufficient material to code for all the amino acids in apoVLDL-II, including the signal peptide (Chan et al., 1980) plus approximately 100 base pairs of noncoding sequence at the 3′ terminus.

After the "apoVLDL-II DNA" clone was obtained, a major fragment of the recombinant DNA was nick-translated and used as a hybridization probe to examine the effect of estrogen on the concentration of apoVLDL-II mRNA sequences. Hormone treatment resulted in a 12 000-fold increase in the intracellular concentration of hepatic apoVLDL-II mRNA sequences within 12 h. This observation indicated that we were studying an estrogen-responsive gene and confirmed our previous studies with translational assays (Chan et al., 1976a, 1980). The successful cloning of the apoVLDL-IIcoding sequence makes possible future detailed studies on the regulation of the apoVLDL-II gene by various hormones. Furthermore, it will permit the identification and analysis of the apoVLDL-II gene present in total cockerel DNA.

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Thermal Dependence of Apolipoprotein A-I–Phospholipid Recombination†

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ABSTRACT: Studies of the recombination of apolipoprotein A-I (apo A-I), the major protein constituent of human high-density lipoprotein, and various synthetic phospholipids, both alone and in mixtures, have been performed. Pure diacyl phospholipids containing homologous fatty acids of the C_{12}, C_{13}, C_{14}, and C_{15} series, as well as the two positional isomers containing C_{14} and C_{16} fatty acids in positions 1 and 2, undergo reaction with the apo A-I protein only near their gel–liquid-crystalline transition temperatures; the degree of reactivity of these phospholipids toward recombination was observed to decrease as the transition temperature increased. The presence of lysolecithin in the incubation mixtures at proportions of 5 mol/mol of protein or lower was not found to have a significant effect on the rate of recombination. Binary mixtures of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine at various proportions react maximally with apo A-I at the onset of the phase transition, as judged by comparison with published phase diagrams; in this case also, the rate of recombination was observed to decline for mixtures with higher phase transition temperatures. These results are interpreted in terms of protein insertion at lattice defects arising from the presence of phospholipid clusters undergoing the phase transition; these clusters are derived from the cooperative and simultaneous melting of a number of molecules, the cooperativity being dependent upon the nature of the phospholipid. It is postulated that phospholipids which melt in a more highly cooperative fashion are more capable of interacting with the apolipoproteins since these phospholipids will possess larger lattice defects during the phase transition.

A great deal of interest has been focused in recent years on the recombination[1] of apolipoproteins, especially apolipoprotein A-I (apo A-I) from the high-density lipoprotein, with synthetic phospholipids. The apo A-I is of particular interest because it is the major protein component of human high-density lipoprotein (Shore & Shore, 1969); the high-density lipoprotein of several other mammalian species (Edelstein et al., 1976; Jonas, 1972; Cox & Tanford, 1968) also contain as virtually their sole protein complement an equivalent apolipoprotein. Apo A-I has also been found to be the major protein component of a disk-shaped high-density particle secreted by rat intestine (Green et al., 1978).

While numerous studies of recombination of apo A-I with well-defined phospholipids have been performed, extensive binding of lipid by apo A-I has only been found for dimyristoylphosphatidylcholine [DMPC, di(14:O)PC][2] (Hauser et al., 1974; Andrews et al., 1976; Middelhoff et al., 1976; Tall et al., 1977; Jonas & Krajnovich, 1977; Pownall et al., 1978; Swaney, 1980a) and for didecanoylphosphatidylcholine [di-(10:O)PC] (Reynolds et al., 1977). Especially relevant to the

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[1] Recombination is used here in the general sense of reformation of a lipid–protein complex, even though the synthetic lipids used here are not found in significant amounts in naturally occurring lipoproteins.

[2] Abbreviations used: DMPC or di(14:O)PC, dimyristoylphosphatidylcholine; DLPC or di(12:O)PC, dilauroylphosphatidylcholine; DPPC or di(16:O)PC, dipalmitoylphosphatidylcholine; di(13:O)PC, di-(tridecanoyl)phosphatidylcholine; di(15:O)PC, di(pentadecanoyl)phosphatidylcholine; PMPC, 1-palmitoyl-2-myristoylphosphatidylcholine; MPPC, 1-myristoyl-2-palmitoylphosphatidylcholine; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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