Rat apolipoprotein C-II lacks the conserved site for proteolytic cleavage of the pro-form

Yvonne Andersson, Lars Thelander, and Gunilla Bengtsson-Olivecrona

Department of Medical Biochemistry and Biophysics, University of Umeå, S-90187 Umeå, Sweden

Abstract Apolipoprotein C-II (apoC-II) plays a critical role in the metabolism of plasma lipoproteins as an activator for lipoprotein lipase. Human apoC-II consists of 79 amino acid residues (pro-apoC-II). A minor fraction is converted to a mature form by cleavage at the site QQDE releasing the 6 amino-terminal residues. We have cloned and sequenced the cDNA for rat apoC-II from a liver cDNA library using human apoC-II cDNA as a probe. The cDNA encodes a protein of 97 amino acid residues including a signal peptide of 22 amino acid residues. There is approximately 60% similarity between the deduced amino acid sequence of rat apoC-II and other apoC-II sequences presently known (human, monkey, dog, cow, and guinea pig). Compared to these, rat apoC-II is one residue shorter at the carboxyl terminus. Furthermore, there is a deletion of 3 amino acid residues (PQQ) in the highly conserved cleavage site where processing from pro- to mature apoC-II occurs in other species. Accordingly, rat apoC-II isolated from plasma was mainly in the pro-form. Northern blot analyses indicated that rat apoC-II is expressed both in liver and in small intestine. —**Andersson, Y., L. Thelander, and G. Bengtsson-Olivecrona.** Rat apolipoprotein C-II lacks the conserved site for proteolytic cleavage of the pro-form. *J. Lipid Res.* 1991. **32:** 1805–1809.

Supplementary key words lipoprotein lipase • proteolytic processing • protein purification • cDNA sequence • apolipoprotein A-I

Apolipoprotein C-II (apoC-II) has a well-known function as an activator for lipoprotein lipase (1, 2). Genetic deficiencies affecting apoC-II lead to massive hypertriglyceridemias, demonstrating the importance of the activation in vivo (3). Human apoC-II is a 79 amino acid residue protein (4, 5). Studies with peptide fragments of apoC-II have demonstrated that the carboxy-terminal third of the molecule contains the structures needed for interaction with lipoprotein lipase and for activation, while the amino-terminal part probably forms amphipathic helices that anchor the protein to the lipid particle (1). The amino acid sequences are now known for apoC-II analogues from several species; monkey, cow, dog, and guinea pig (6–9). The conservation is high in the carboxy-terminal part of the apoC-II molecules, while there are more substitutions in the amino-terminal portion. This probably reflects that the lipid-binding function has less specific structural requirements. There is, however, one area in the amino-terminal end that is fully conserved in all apoC-II sequences known this far. This is the QQDE-sequence (residues 5 to 8 in the human sequence). Fojo et al. (10) reported that a small fraction of human plasma apoC-II was proteolytically processed at this site. Later, processing of both monkey apoC-II (11) and bovine apoC-II (8) was reported. In these species the cleaved form of apoC-II was dominant in plasma. Both forms of apoC-II stimulate lipoprotein lipase in in vitro systems (8, 10, 11). Thus, the function of the cleavage, if any, is not known.

We were interested in studying the processing of apoC-II in an animal model. For that we turned to rats, because their lipoprotein metabolism has been extensively studied and their lipoprotein lipase system is well known. However, the primary structure of rat apoC-II was not known. We therefore determined this from the corresponding cDNA which we cloned using the cross-hybridizing human apoC-II cDNA as a probe. To our surprise, in rat apoC-II there is a deletion of 3 amino acid residues, which partly affects the proteolytic cleavage site. This suggested that no cleavage of apoC-II occurs in this animal. To directly study this, rat apoC-II was isolated from plasma and its amino-terminal sequence was determined.

METHODS

Cloning and sequencing of rat apoC-II cDNA

Preparation of RNA, Northern blot analyses, construction of cDNA library, and DNA sequencing was done as previously described for guinea pig apoC-II (9). The cDNA library was made from 5 μg poly(A) RNA from rat liver. About 60,000 colonies were screened with the human apoC-II cDNA probe. One positive clone, containing a rat cDNA insert of about 600 base pairs, was obtained. This size agreed well with the mRNA size obtained on Northern blots of RNA from rat liver and intestine. The DNA sequencing was interrupted after nucleotide 244, probably due to secondary structure. A synthetic oligo-

nucleotide, 5'GGACATGTACAGCAA3' (Symbicom, Umeå, Sweden), corresponding to nucleotide 224 to 238 was therefore used as a primer for the sequence reaction. Rat apoC-II cDNA was prepared and used as a probe in Northern blot analyses of RNA from rat liver and intestine as was previously described for guinea pig apoC-II (9).

Isolation of rat apoC-II

ApoC-II was isolated from 110 ml rat plasma by adsorption to a lipid emulsion (Intralipid), flotation of the lipid droplets, and delipidation of the lipid-bound proteins with ethanol-diethyl ether, as previously described for isolation of apoC-II from bovine and guinea pig plasma (8, 9). After solubilization in 3 M urea (10 mg/ml), the proteins were separated by polyacrylamide gel electrophoresis in alkaline urea or by isoelectric focusing on Immobiline Dry Plates, pH range 4.2–4.9 (Pharmacia LKB Biotechnology). Running conditions, extraction procedures for detection of apoC-II in gel slices, transfer of the proteins to Immobilon filters, and amino acid sequence analyses were as described previously (9).

RESULTS

Fig. 1 shows the nucleotide sequence of rat apoC-II cDNA with the deduced amino acid sequence. The cDNA encodes a protein of 97 amino acid residues, including a signal peptide of 22 amino acid residues. There is a 17 nucleotide 5'-untranslated region, and a 3'-untranslated region containing 168 nucleotides. The sequence of 479 nucleotides plus the poly(A) tail agrees well with the observed size of the apoC-II mRNA transcript on Northern blots. The deduced amino acid sequence for rat apoC-II is compared to all other presently known apoC-II sequences in **Fig. 2.** The signal sequence for rat apoC-II is very similar to corresponding sequences in humans, monkey, and dog. The signal sequence for guinea pig apoC-II differs both in length and structure, particularly in its first half. There are two areas of particular interest in the sequence of rat apoC-II. In the carboxyl terminus, rat apoC-II is one residue shorter than the other apoC-II variants. In the amino terminus there are marked differences between the rat sequence and the others, both in charge and in structure. There is a deletion of 3 amino acid

```
  1 CGGCACGAGCAGACAGC

 18 ATGGGGTCTCGTTTCTTCCTGGCTCTATTCCTGGCTCTCCTAGTGTTGGGA
    M  G  S  R  F  F  L  A  L  F  L  A  L  L  V  L  G
-22

 69 AACGAGGTCCAGGGGACCGAGGAAGATGATCCGGGCAGCTCAGCTCTGCTG
    N  E  V  Q  G  T  E  E  D  D  P  G  S  S  A  L  L
                  1

120 GACACGGTACAGGAGCACTTGTTCAGTTACTGGAACTCTGCCAAGGCGGCC
    D  T  V  Q  E  H  L  F  S  Y  W  N  S  A  K  A  A

171 GCCGGAGAACTATACCAGAAGACATACCTGACCAGCGTGGACGAGAAACTG
    A  G  E  L  Y  Q  K  T  Y  L  T  S  V  D  E  K  L
      ..............
222 AGGGACATGTACAGCAAAAGCTCGGCGGCCATGACCACGTACGCTGGCATT
    R  D  M  Y  S  K  S  S  A  A  M  T  T  Y  A  G  I

273 TTTACAGACCAGCTCCTTACTCTCCTGAAAGGAGAGTAG
    F  T  D  Q  L  L  T  L  L  K  G  E  stop

312 TTACTACATAAACTCCGAGTTAGGGGAAAGGAAAACCGGACACCCGGCCCT
363 TTTGCTCTCATCACCTGACCAGGATGGTCCTACATCTCCCTGTCCCAGCAT
414 CTCCCATCTTCCTGCGTCAACTCTAGACCAAATTCTTTCAATGAAAGAATA
465 AATGAGTCCAGTTTC-(poly A)
```

Fig. 1. Nucleotide sequence of the cDNA encoding rat apoC-II and the predicted amino acid sequence of the protein. The nucleotide sequence begins with the 5'-untranslated region and is numbered to the left of each line. The first amino acid in the signal peptide (**-22**) and the first amino acid in the apoC-II protein (**1**) are numbered. The positions of the stop codon and the polyadenylation signal are underlined. The site of the synthetic oligonucleotide used for sequencing is marked with dots.

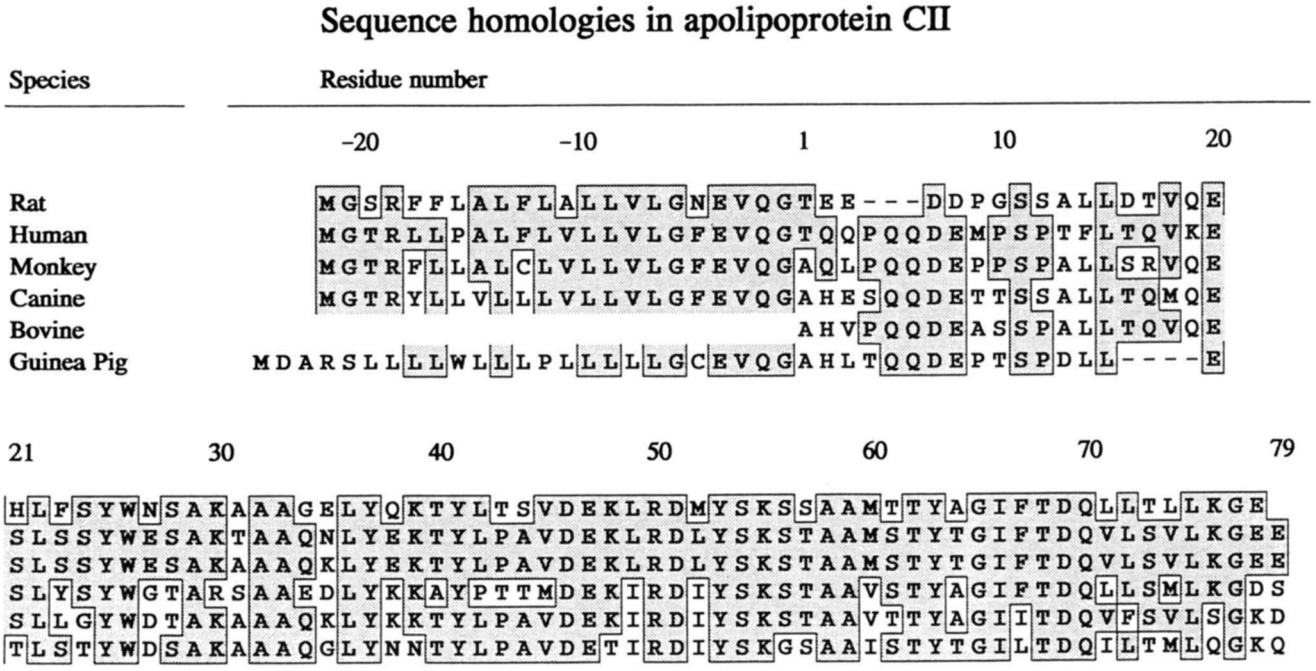

Fig. 2. Comparison of the deduced amino acid sequence of rat apoC-II to all other apoC-II sequences presently known. Negative numbers are used for amino acid residues in the signal peptides. Deletions in rat apoC-II (three residues) and in guinea pig apoC-II (four residues) are indicated by ---. Shaded boxes indicate homologies to the human sequence.

residues, corresponding to residues 4–6 in the human sequence. This affects the highly conserved sequence, QQDE, which is found in apoC-II from all other species investigated so far. Furthermore, the amino terminus of rat apoC-II is negatively charged because of the two glutamic acid residues in positions 2 and 3. In the canine sequence there is one glutamic acid residue in position 3, but the other variants are uncharged or contain histidine residues.

The lack of the cleavage sequence raised the question whether rat apoC-II was cleaved at some other site instead. We therefore isolated apoC-II from rat plasma to enable determination of its amino-terminal sequence. For this, lipid-binding proteins in plasma were isolated by adsorption to a lipid emulsion, a method previously used for isolation of apoC-II from bovine and guinea pig plasma. Polyacrylamide gel electrophoresis in alkaline urea of the delipidated proteins showed three rapidly migrating bands (**Fig. 3a**). On extraction of gel slices, apoC-II-like activity was found in one symmetrical peak associated with the upper two of these three bands. Amino-terminal sequence analyses of the slowest and the fastest of the three protein bands revealed the sequence Asp-Glu-Gly-Glu-Gly- for both. This sequence is identical to the amino-terminal sequence of rat apolipoprtoein C-III (12). To improve the separation of the activator protein from the isoforms of apoC-III, the material was subjected to isoelectric focusing in a narrow pH gradient in the pres-

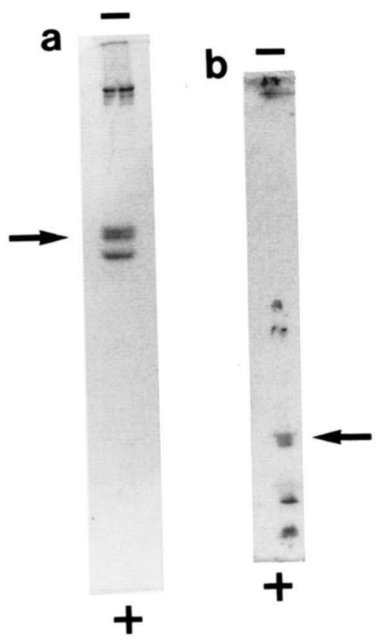

Fig. 3. Separation of lipid binding proteins from rat plasma by electrophoresis and isoelectric focusing. Panel a: lipid binding proteins from rat plasma (about 30 μg) separated by polyacrylamide gel electrophoresis in alkaline urea (9). Panel b: the same material as in panel a (about 100 μg) was separated by isoelectric focusing in the pH range 4.2–4.9. Minus and plus signs indicate the positions of the cathode and the anode, respectively. Corresponding unstained lanes were cut in 2-mm slices, extracted, and assayed for their ability to stimulate lipoprotein lipase activity as described previously (9). The arrows indicate the position of the peak of lipase stimulation.

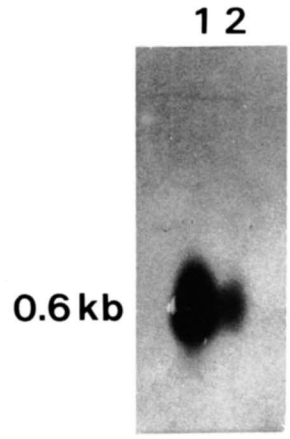

Fig. 4. Northern blot analyses of RNA from rat liver and intestine. Lane 1: total RNA from liver (20 μg). Lane 2: total RNA from intestine (20 μg). Rat apoC-II cDNA was used as a probe. The X-ray film was exposed to the filter for 4 h at −70°C on an intensifying screen. Hybridization with a rat β-actin cDNA probe was done to verify that the RNA preparations were intact (not shown).

ence of urea (Fig. 3b). On this gel apoC-II-like activity was found associated with a well-separated, although not well-concentrated protein band. Amino-terminal sequence analyses in five cycles of the acidic and the basic edges of this band revealed the same sequence, Thr-Glu-Glu-Asp-Asp-. This was identical with the expected amino-terminal sequence of rat apoC-II.

Northern blot analyses of total RNA from rat liver and small intestine indicated expression in both tissues (**Fig. 4**). Scanning of the film indicated that the level of expression in the intestine was approximately 15% of that in liver.

DISCUSSION

The aim of this study was to determine the primary structure for rat apoC-II. A characterization of the low molecular weight proteins of rat high density lipoproteins was done by Herbert et al. in 1974 (13). They reported the presence of two isoforms of apolipoprotein C-III (apoC-III), and designated them apoC-III-0 and apoC-III-3 based on the content of sialic acid. On electrophoresis in alkaline urea and on chromatography on DEAE-cellulose, rat apoC-II partly comigrated with apoC-III-0. The authors were, however, able to determine both the amino terminal and the carboxy-terminal amino acid residues of rat apoC-II, data that are now confirmed by our nucleotide sequence. Our pattern on alkaline urea gels was similar to that obtained by Herbert et al. (13), but we resolved three bands whereas they resolved two. Amino-terminal sequence analyses indicated that the middle band was apoC-II, since the slower and the faster migrating bands were isoforms of apoC-III. Swaney and Gidez (14) had demonstrated separation between rat apoC-II and the apoC-III isoforms by isoelectric focusing. We therefore chose a narrow pH gradient for isolation of rat apoC-II to determine the amino-terminal sequence of the plasma form and to confirm the sequence deduced from the cDNA. From extraction of apoC-II-like activity from gel slices, by the methodology previously used for apoC-II variants from cow and guinea pig, we could conclude that there is only one major isoform of rat apoC-II. A proteolytically processed form, e.g., cleaved at the Glu-Asp bond, would probably lack several negative charges, and would separate from the pro-form in the systems used. We cannot, however, exclude the possibility that a minor fraction of rat apoC-II could be processed, but not detected in our assay.

The conserved sequence QQDE has been found in all other apoC-II analogues that have been sequenced until now (5–9) and also in pro-apoA-I analogues from several mammals (15–22). There is, however, some sequence variation. Rabbit pro-apoA-I has QRDE (23) and chickens have QHDE (24). Nonetheless, both proforms are cleaved (24, 25). Neither for apoA-I nor for apoC-II has a functional role for the cleavage been found. Both proteins function as activators for enzymes: apoA-I for lecithin: cholesterol acyltransferase (26) and apoC-II for lipoprotein lipase (1, 2). It is clear that this function is not dependent on the proteolytic processing (8, 10, 11). For apoC-II this fits with the knowledge that, at least in vitro, a short part of the carboxy-terminal end of the molecule is sufficient for full activity (1). Similarly, carboxy-terminal fragments of apoA-I stimulate lecithin:cholesterol acyltransferase (27). Thus, clues to the role of the processing must be sought in other areas of function of these proteins, e.g., regulatory effects on lipoprotein-receptor interactions.

We thank Solveig Nilsson for excellent technical assistance and Dr. Per-Ingvar Ohlsson for the amino acid sequence analyses. We also thank Prof. Steve Humphries, Charing Cross Sunley Research Centre, Hammersmith, London, England for providing the human apoC-II cDNA probe. This work was supported by grants from the Swedish Medical Research Council (B13X-727), from the Swedish Cancer Society (2515-B89-02XB), and from the Swedish Natural Science Research Council.

Manuscript received 15 May 1991 and in revised form 21 August 1991.

REFERENCES

1. Smith, L. C., and H. J. Pownall. 1984. Lipoprotein lipase. *In* Lipases. B. Borgström and H. Brockman, editors. Elsevier/North Holland Biomedical, Amsterdam. 263–305.
2. Olivecrona, T., and G. Bengtsson-Olivecrona. 1987. Lipoprotein lipase from milk—the model enzyme in lipoprotein lipase research. *In* Lipoprotein Lipase. J. Borensztajn, editor. Evener Publishers, Chicago. 15–58.

3. Breckenridge, W. C., J. A. Little, G. Steiner, A. Chow, and M. Poapst. 1978. Hypertriglyceridemia associated with deficiency of apolipoprotein C-II. *N. Engl. J. Med.* **298:** 1265–1273.
4. Hospattankar, A. V., T. Fairwell, R. Ronan, and H. B. Brewer, Jr. 1984. Amino acid sequence of human plasma apolipoprotein C-II from normal and hyperlipoproteinemic subjects. *J. Biol. Chem.* **259:** 318–322.
5. Wei, C-F., Y-K. Tsao, D. L. Rodberson, A. M. Gotto, Jr., K. Brown, and L. Chan. 1985. The structure of the human apolipoprotein C-II gene. Electron microscopic analysis of RNA:DNA hybrids, complete nucleotide sequence, and identification of 5' homologous sequences among apolipoprotein genes. *J. Biol. Chem.* **260:** 15211–15221.
6. Whitted, B. E., C. K. Castle, H. G. Polites, G. W. Melchior, and K. R. Marotti. 1989. Purification, cloning and nucleotide sequence determination of cynomolgus monkey apolipoprotein C-II: comparison to the human sequence. *Mol. Cell. Biochem.* **90:** 69–79.
7. Datta, S., W-H. Li, I. Ghosh, C-C. Luo, and L. Chan. 1987. Structure and expression of dog apolipoprotein C-II and C-III mRNAs. Implications for the evolution and functional constraints of apolipoprotein structure. *J. Biol. Chem.* **262:** 10588–10593.
8. Bengtsson-Olivecrona, G., and K. Sletten. 1990. Primary structure of the bovine analogues to human apolipoproteins C-II and C-III. Studies on isoforms and evidence for proteolytic processing. *Eur. J. Biochem.* **192:** 515–521.
9. Andersson, Y., L. Thelander, and G. Bengtsson-Olivecrona. 1991. Demonstration of apolipoprotein C-II in guinea pigs. Functional characteristics, cDNA sequence, and tissue expression. *J. Biol. Chem.* **266:** 4074–4080.
10. Fojo, S. S., L. Taam, T. Fairwell, R. Ronan, C. Bishop, M. S. Meng, J. M. Hoeg, D. L. Sprecher, and H. B. Brewer, Jr. 1986. Human preapolipoprotein C-II. Analysis of major plasma isoforms. *J. Biol. Chem.* **261:** 9591–9594.
11. Herbert, P. N., L. L. Bausserman, K. M. Lynch, A. L. Saritelli, M. A. Kantor, R. J. Nicolosi, and R. S. Shulman. 1987. Homologues of the human C and A apolipoproteins in the *Macaca fascicularis* (cynomolgus) monkey. *Biochemistry.* **26:** 1457–1463.
12. Haddad, I. A., J. M. Ordovas, T. Fitzpatrick, and S. K. Karathanasis. 1986. Linkage, evolution, and expression of the rat apolipoprotein A-I, C-III, and A-IV genes. *J. Biol. Chem.* **261:** 13268–13277.
13. Herbert, P. N., H. G. Windmueller, T. P. Bersot, and R. S. Shulman. 1974. Characterization of the rat apolipoproteins. 1. The low molecular weight proteins of rat plasma high density lipoproteins. *J. Biol. Chem.* **249:** 5718–5724.
14. Swaney, J. B., and L. I. Gidez. 1977. Analysis of rat serum apolipoproteins by isoelectric focusing. II. Studies on the low molecular weight subunits. *J. Lipid Res.* **18:** 69–76.
15. Gordon, J. I., H. F. Sims, S. R. Lentz, C. Edelstein, A. M. Scanu, and A. W. Strauss. 1983. Proteolytic processing of human preapolipoprotein A-I. A proposed defect in the conversion of pro A-I to A-I in Tangier's disease. *J. Biol. Chem.* **258:** 4037–4044.
16. Zannis, V. I., S. K. Karathanasis, H. T. Keutmann, G. Goldberger, and J. L. Breslow. 1983. Intracellular and extracellular processing of human apolipoprotein A-I: secreted apolipoprotein A-I isoprotein 2 is a propeptide. *Proc. Natl. Acad. Sci. USA.* **80:** 2574–2578.
17. Sliwkowski, M. B., and H. G. Windmueller. 1984. Rat liver and small intestine produce proapolipoprotein A-I which is slowly processed to apolipoprotein A-I in the circulation. *J. Biol. Chem.* **259:** 6459–6465.
18. Stoffel, W. 1984. Synthesis, transport, and processing of apolipoproteins of high density lipoproteins. *J. Lipid Res.* **25:** 1586–1592.
19. Scanu, A. M., R. E. Byrne, and C. Edelstein. 1984. Proteolytic events affecting plasma apolipoproteins at the co- and post-translational levels and after maturation. *J. Lipid Res.* **25:** 1593–1602.
20. Polites, H. G., G. W. Melchior, C. K. Castle, and K. R. Marotti. 1986. The primary structure of cynomolgus monkey apolipoprotein A-I deduced from the cDNA sequence: comparison to the human sequence. *Gene.* **49:** 103–110.
21. Hixon, J. E., S. Borenstein, L. A. Cox, D. L. Rainwater, and J. L. VandeBerg. 1988. The baboon gene for apolipoprotein A-I: characterization of a cDNA clone and identification of DNA polymorphism for genetic studies of cholesterol metabolism. *Gene.* **74:** 483–490.
22. O'Huigin, C., L. Chan, and W. H. Li. 1991. Cloning and sequencing of bovine apolipoprotein A-I cDNA and molecular evolution of apolipoproteins A-I and B-100. *Mol. Biol. Evol.* In press.
23. Pan, T., Q. Hao, T-T. Yamin, P-H. Dai, B. Chen, S. Chen, P. A. Kroon, and Y. Chao. 1987. Rabbit apolipoprotein A-I mRNA and gene. Evidence that rabbit apolipoprotein A-I is synthesized in the intestine but not in the liver. *Eur. J. Biochem.* **170:** 99–104.
24. Rajavashisth, T. B., P. A. Dawson, D. L. Williams, J. E. Shackelford, H. Lebherz, and A. J. Lusis. 1987. Structure, evolution, and regulation of chicken apolipoprotein A-I. *J. Biol. Chem.* **262:** 7058–7065.
25. Yang, C., T. Yang, H. J. Pownall, and A. M. Gotto, Jr. 1986. The primary structure of apolipoprotein A-I from rabbit high-density lipoprotein. *Eur. J. Biochem.* **160:** 427–431.
26. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46:** 1493–1498.
27. Sparrow, J. T., and A. M. Gotto. 1980. Phospholipid binding studies with synthetic apolipoprotein fragments. *Ann. N.Y. Acad. Sci.* **348:** 187–212.