Mice have a monodisperse high density lipoprotein (HDL) profile, whereas humans have two major subfractions designated HDL$_2$ and HDL$_3$. Human apoA-I transgenic mice exhibit a human-like HDL profile, indicating that the amino acid sequence of apoA-I is a determinant of the HDL profile. Comparison of the primary sequence of mouse and human apoA-I and the previously designated “hinge” domain of apoA-I led us to hypothesize that α-helices 7 and 8 (7/8) are determinants of HDL subclass distribution. The following proteins were expressed in _Escherichia coli_: human apoA-I, T7-hAI; mouse apoA-I, T7-mAI; chimeric human apoA-I containing murine helices 7/8 in place of human helices 7/8, T7-hAI(m7/8); and the reciprocal chimera, T7-mAI(h7/8). The recombinant proteins were examined for their association with human plasma HDL subclasses. The results demonstrated that T7-hAI bound HDL$_2$ and HDL$_3$ equally well, whereas T7-mAI bound to HDL$_2$ preferentially. T7-hAI(m7/8) behaved like T7-mAI, and T7-mAI(h7/8) behaved like T7-hAI. Thus, α-helices 7/8 are strong contributors to the pattern of HDL subclass association. Self-association, α-helicity, cholesterol efflux, and lecithin-cholesterol acyltransferase activity of the recombinant proteins were also assessed. Human apoA-I self-associates more and activates human lecithin-cholesterol acyltransferase better than mouse apoA-I. These differential characteristics of human and mouse apoA-I are not dependent on helices 7/8.

Plasma levels of high density lipoprotein (HDL) are inversely correlated with the development of coronary artery disease. Human and non-human primate HDL are heterogeneous, with the two major subclasses designated HDL$_2$ and HDL$_3$. Epidemiological data suggest that HDL$_2$ is more atheroprotective than HDL$_3$ (1). The epidemiological data and _in vitro_ assays in which HDL subclasses have discrete functional capacities (2) suggest that HDL subclasses may have discrete atheroprotective mechanisms. In contrast to primates, most other mammals have less polydisperse HDL. In the case of the mouse, a species being intensively studied with molecular genetic tools in atherosclerosis research, the HDL size and density resemble human HDL$_2$. A major determinant of HDL size and density is the structure of the major HDL protein, apoprotein A-I (apoA-I). This is exemplified by human apoA-I transgenic C57BL/6 mice that have a human-like HDL profile (3).

ApoA-I is a 243-amino acid protein that can be divided into an N-terminal globular domain (residues 1–43), encoded by exon 3 of the apoA-I gene, and a C-terminal domain (residues 44–243), encoded by exon 4 of the gene (4). The C-terminal domain is predicted to contain eight 22-mer and two 11-mer amphipathic α-helices, with most of the helices being punctuated by prolines. Functionally, apoA-I activates LCAT (5) and promotes cellular cholesterol efflux (6) and is a ligand for SR-BI and ABC A-1 (7, 8). Furthermore, apoA-I is believed to be the structural framework that mediates changes in HDL size (9). Studies have suggested that helices 6 and 7 are critical for maximal activation of LCAT (10–12), that helices 4–7, the putative hinge domain, are necessary for accommodating changes in HDL size (13, 14), and that helices 1, 9, and 10 are essential for initial lipid binding (15, 16).

A previous study (17) from this laboratory using proteins chimeric for human apoE and apoA-I demonstrated that residues 44–243 of apoA-I, which include the repeating amphipathic helical domain, is most influential in subclass determination. A sequence comparison of the helical domain of mouse and human apoA-I and the previously reported hinge domain led us to focus on helices 7/8 (residues 165–209) as the most likely region to influence the distinct human and mouse HDL subclass distributions. This was explored using chimeric apoprotein molecules in which helices 7/8 are exchanged between the human or mouse apoA-I proteins.

**EXPERIMENTAL PROCEDURES**

Elements Common to All Recombinant ApoA-I cDNAs—Site-directed mutagenesis (Bio-Rad T7 in Vitro Mutagenesis) and PCR amplification/mutagenesis, using the oligonucleotides (oligos) shown in Table I, were utilized to construct recombinant apoA-I cDNAs. The relative position of restriction sites used to generate the mature apoA-Is and chimeras are shown in Fig. 1. All DNAs were sequenced by the method of Sanger et al. (18) or by fluorescent sequencing at the DNA Sequencing Core Facility at the University of Chicago. The mature apoA1 cDNAs were subcloned into the pET 28c+ vector (Novagen), a bacterial expression vector containing a His$_6$-T7 epitope tag sequence (MGSSHHHHHH-SGLVPRGSHMASMTGQGQMG)R. The His$_6$ was used to purify recom-
binant apoA-I, and the T7 epitope was used for detection in Western blotting. The recombinant cDNAs were subcloned downstream and in frame with this tag by utilizing the BamHI site in the polylinker region. This introduced an additional N-terminal dipeptide (IQ) between the His6-T7 epitope tag and mature apoA-I sequence when the proteins were expressed in the pET 28C/H11001 vector.

**Construction of Mature Human ApoA-I cDNA**
- The 840-base pair prepro-apoA-I in the XbaI site of pCMV4 (17) was subcloned into the corresponding site of M13mp19. Single strand mutagenesis using Oligo 1 was used to create a BamHI restriction site between the prepro-sequence and mature apoA-I. The 782-bp fragment encoding the mature protein was excised with BamHI-HindIII and subcloned into the corresponding sites of pET 28c/H11001.

**Construction of Mature Mouse ApoA-I cDNA**
- Reverse transcription-PCR of total mouse liver RNA was used to obtain mouse apoA-I cDNA sequence. The 5' oligonucleotide (Oligo 2) contained a HindIII site and the 3' oligonucleotide (Oligo 3) contained a BamHI site, allowing subcloning of the mouse apoA-I cDNA into the corresponding regions of M13mp19. Oligo 4 was used to create a BamHI restriction site between the codons for the pro-sequence and the mature apoA-I sequence. The 782-bp fragment encoding the mature protein was excised with BamHI-HindIII and subcloned into the corresponding sites of pET 28c/H11001.

### Table I

**Synthetic oligonucleotides used for the construction of cDNAs encoding human and mouse apoA-I and the chimera apoA-Is**

| Use of oligonucleotide | ApoA-I cDNA location nt | Sequence |
|------------------------|-------------------------|----------|
| hAI Mutagenesis 1. Mature hAI (+B) | 70 | 5' gct cgg cat ttc tgg atc caa gat gaa ccc |
| mAI PCR amplification 2. 5' mAI | 883 (complement) | 5' ata aga ttt gtc gga gag cgt ceg ggg a |
| | 3' mAI | 93 (complement) | 5' atg gat ceg ctt att gta aag aag cca at |
| | 7. Mature mAI (+B) | 93 (complement) | 5' ctg ggg ttc atg cct cca tca ctc cca |
| T7-hAI(m7/8) Excise h7/8 (mutagenesis) 5. Human apoA-I (+A) | 556 | 5' gae ggt ceg ceg aag ceg ctt gce ccc tac age gac |
| | 6. Human apoA-I (+A + P) | 712 | 5' aag gce aac ccc ggg ceg cgg gac cgg caa gg |
| | 7. M13mp19 (−P) Polylinker (complement) | 735 (complement) | 5' tcc tct aga gtc gac ctt ggt cgt cca |
| T7-mAI(h7/8) Excise m7/8 (mutagenesis) 8. Mouse apoA-I (−EO) | 383 | 5' tcc tct agg tct tct ccc tcc at |
| | h 7/8 insert (PCR mutagenesis) 9. 5' human 7/8 (+N) | 573 | 5' att aag ctt ceg cta gce ccc tac age gac gag |
| | 10. 3' human 7/8 | 735 (complement) | 5' atg gat ceg cgg agg tcc tcg agc |

Fig. 1. Amino acid sequence alignment of human (hu) and mouse (mo) apoA-I. The apoA-Is are divided into the prepro sequence, non-helical sequence, and helices 1–10. The first amino acid position in each segment (based on human numbering) is shown. The relative positions of the restriction endonuclease sites used in this study are identified by arrowheads (downward facing, human apoA-I; upward facing, mouse apoA-I). The shaded region represents the amino acid sequence that is exchanged between the human and mouse chimeras. Only non-identical residues are shown for mouse apoA-I. Boldface type in the mouse sequence represents changes in charge/proline punctuation. Hydrophobic changes are marked with * above. Deletions are marked with −. Abbreviations used are as follows: B, BamHI; H, HindIII; EO, EcoO1091; N, NheI; A, AvrII; and P, PstI.
mature mouse apoA-I cDNA was excised with BamHI and placed in the corresponding region of pET 28c + vector.

Construction of Mature T7-T7-hAl(m7/8) Chimera cDNA—The T7-T7-hAl(m7/8) chimera was prepared by excising the cDNA segment encoding human residues 163–212 and replacing it with the corresponding amino acid residues. Next, the cDNA—coding residues 163–212, Glu162, and Asp213 (CCT GAC GAC) was mutagenized to CCT GCA GAC (Oligo 6), introducing a PstI site as well as changing the corresponding amino acid sequence to Pro162, Ala162, and Asp213. After removing the PstI site in the polylinker region of M13mp19 (Oligo 7), the human apoA-I cDNA was linearized at the remaining unique PstI, the 3' overhang removed with mung bean nuclelease and the construct digested with XbaI, excising the cDNA fragment encoding human residues 163–212. The cDNA encoding the corresponding murine residues (Fig. 1) was obtained by digesting the mature mouse apoA-I cDNA with Eco109I, blunt-ending the 5' overhang with mung bean nuclelease, and digesting with NheI. As NheI and XbaI have compatible overhangs, the 163–212-residue murine fragment was ligated into the corresponding XbaI site in the vector containing human apoA-I. The T7-hAl(m7/8) insert was excised with BamHI-HindIII and ligated into the corresponding region of the pET 28c + vector.

Construction of Mature T7-mAl(h7/8) Chimera cDNA—To generate T7-mAl(h7/8) cDNA, the codon for residue 88 in mouse apoA-I was mutated to ablate an Eco109I site using Oligo 8, leaving a unique Eco109I that cuts between the codons for murine residues 212 and 213 (based on the human apoA-I sequence). The modified cDNA was digested with NheI, which cleaved between residues 162 and 163, and Eco109I to remove the cDNA encoding murine residues 163–212. The corresponding region of human apoA-I was obtained by PCR. The 5'-oligomer (Oligo 9) contained a 5'-flanking HindIII site and generated an NheI site to cleave between residues 162 and 163. The generation of the NheI site changed His162 to Gln162. The 3'-oligomer (Oligo 10) contained a BamHI site after the codon for human residue 215. The PCR fragment was subcloned into the HindIII/BamHI site of pUC19. The cDNA encoding residues 163–212 was excised with NheI and Eco109I and subcloned into the corresponding region in mouse apoA-I, yielding the T7-mAl(h7/8) chimeric cDNA sequence. Note that the codon for human residue 162, which was mutated to Gln and included in the final chimeric construct, is in helix 6. In mouse helix 6 residue 162 is a Glu so that this insert reconstitutes the wild-type murine sequence in this helix. The chimeric cDNA was excised with BamHI and placed in the corresponding region of the pET 28c + vector.

Expression and Purification of His-T7 ApoA-I Recombinant Proteins—The recombinant apoA-I PET expression vectors in E. coli BL21(DE3) pLysS (Novagen) were expressed and purified as described previously (19). Purified apoA-I was dialyzed against Standard buffer, pH 8.0, and subjected to 10% SDS-PAGE gels and the protein bands were visualized by ECL (Amersham Biosciences and Molecular Dynamics). Density measurements were determined from densitometric scanning. Visualized by ECL (Amersham Biosciences and Molecular Dynamics), and the proportion of apoA-I in HDL 2 and HDL3 was determined using ImageQuant software (Genomic Solutions Inc.), and the proportion of apoA-I in HDL 2 and HDL3 was determined using ImageQuant software (Genomic Solutions Inc.). The relative amount of recombinant apoA-I, expressed as the percent of total area was determined from densitometric scanning. The relative amount of recombinant apoA-I, expressed as the percent of total area was determined from densitometric scanning. The relative amount of recombinant apoA-I, expressed as the percent of total area was determined from densitometric scanning.
relative amount of HDL_{2} and HDL_{3}, in the total lipoprotein samples used for the association assays. Antibodies that cross-react with mouse apoA-I were removed by passing the antiserum over an Affi-Gel 15 column containing murine HDL. While this human apoA-I-specific antibody recognizes endogenous apoA-I as well as recombinant T7-hAI and T7-mAl(m7/8), the recombinant proteins have a slightly slower mobility on the SDS-acrylamide gels due to the presence of the N-terminal tag. In addition, the recombinant proteins are present at 60-fold lower concentration than the endogenous protein, and thus detection of these proteins in the immunoblots of the HDL fractions requires overexposure of the film.

Concentration-Dependent Binding of Recombinant ApoA-I to HDL—Increasing amounts of recombinant apoA-I (10–80 μg) were added to isolated HDL_{2} or HDL_{3} (200 μg of total protein) in 545 μl of Standard buffer, pH 8.0. After a gentle vortex, 45 μl (designated the reserved incubation mixture) was removed for subsequent generation of standard curves. The remaining 500 μl was incubated at 4 °C for 30 min. Protein aggregates were pelleted by centrifugation at 14,000 × g for 10 min at 4 °C, and the supernatant was applied to two tandem Superose 6 columns (Amersham Biosciences and Molecular Dynamics) that had been equilibrated with Standard buffer, pH 8.0. HDL and free proteins were eluted at room temperature at a flow rate of 0.8 ml/min. All samples were immediately frozen or placed on ice when they were collected to minimize proteolysis or HDL reformation. Samples to be electrophoresed on 4–20% non-denaturing gradient gels (see below) had the preservative mixture added to them and were stored at 4 °C, for no longer than 4 days, until electrophoresis was performed.

Recombinant and endogenous apoA-Is were quantitated by Western blotting as described above. The reserved incubation mixture was used to generate standard curves for recombinant (range 1.25–20 ng) and endogenous (range 6.25–20 ng) apoA-I, respectively. A standard curve for the appropriate apoprotein was included on every blot to correct for variability in electrophoretic transfer and antibody reactivity. The concentration of recombinant apoA-I in the peak HDL fractions (bound), free protein fractions, and aggregated pellet was determined by linear regression analysis of recombinant or endogenous apoA-I standard. The concentration of bound apoA-I versus free apoA-I was analyzed by Scatchard analysis using Kaleidagraph (version 3.5) software.

Non-denaturing Gradient Gel Electrophoresis—Peak HDL_{2} and HDL_{3} fractions were separated on non-denaturing 4–20% gradient acrylamide gels (NOVEX) by electrophoresis at 125 V/gel for 5 h at 4 °C, according to manufacturer’s instructions. HDL sizes were determined as described previously (23) using 4–30% non-denaturing gradient acrylamide gels (obtained from David Rainwater, Southwest Foundation, San Antonio, TX). The calibration standards (high molecular weight electrophoresis calibration kit) were purchased from Amersham Biosciences.

Statistical Analysis—Results are expressed as means ± S.D. Group differences were tested by analysis of variance and the Scheffe and Tukey/Kramer post-hoc tests, using StatView (version 5.0.1) software.

RESULTS

SDS-PAGE and Western Blot Analysis—Our strategy has involved the synthesis of recombinant apoA-I constructs each containing N-terminal tags consisting of a His tag for purification and a T7 tag for selective identification. The recombinant proteins had the expected molecular weights as determined by SDS-PAGE (Fig. 2) and by electron spray mass spectrometry (data not shown). From densitometric scanning of the gel, the purity of the recombinant proteins was 95%. The remaining 5% of the protein contained a distinct band at 41 kDa. The 41 kDa was not recognized by anti-T7 or polyclonal antibodies directed against human and mouse apoA-I (data not shown), suggesting that this band was a bacterial protein. Recombinant apoA1 purified using the same system also has a 41-kDa band, supporting the suggestion that it was a contaminating bacterial protein. Modification of the washing and elution conditions did not eliminate this band. Because it composed less than 5% of total protein, did not associate with HDL, and did not interfere with apoA-I structure/function (demonstrated below), no further attempts were made to eliminate this band.

Self-association of Lipid-free ApoA-Is—Prior to examining

\[2\] T. Biswas, personal communications.
ApoA-I and HDL Subclass Distribution

Recombinant ApoA-I Secondary Structure—The α-helical content of lipid-free and lipid-bound recombinant apoA-Is was assessed by circular dichroism. The α-helical content of plasma-derived human apoA-I was 40 and 68% for lipid-free and lipid-bound apoA-I, respectively, in agreement with published values (35). The α-helical contents, expressed as the percentage of total amino acids, of lipid-free and lipid-bound recombinantapoA-Is were similar to the respective values for plasma-derived human apoA-I, although the values tended to be slightly lower (Table II). The N-terminal extension of recombinant apoA-I, which does not form an α-helix but is included in the molecular weight used for the α-helical content calculation, could account for the slightly lower α-helical content observed. If the 33 residues of the N-terminal extension are excluded from the calculation of percent α-helical content in the lipid-free state, then the helical content of the recombinant proteins is between 37 and 44%. Thus, recombinant and human plasma apoA-Is have very similar secondary structures.

Functional Properties of Recombinant ApoA-I—The ability of recombinant T7-hAI to activate human LCAT was similar to that of plasma-derived human apoA-I (Table III). Interestingly, T7-mAI:rHDL consistently displayed somewhat lower LCAT activation (79%) as compared with plasma-derived human apoA-I, consistent with previous reports (34) that plasma-derived mouse apoA-I has a lower capacity compared with human apoA-I, T7-hAI, or T7-mAI in the lipid-free or lipid-bound state. Of the T7-mAI that bound to HDL, 66% bound preferentially to HDL2 compared with HDL3 (Figs. 5B and 6). With the chimera T7-hAI(m7/8), 62% of the T7-mAI distributed to the HDL subclasses in a pattern that closely resembles that of T7-hAI than T7-mAI. Conversely, 45 and 55% of T7-mAI(h7/8) distributed to HDL2 and HDL3, re-

As a second assessment of apoA-I function, the capacity of T7-hAI and T7-mAI to promote efflux of cholesterol from radiolabeled fibroblasts was explored. In control incubations, plasma-derived human apoA-I in the context of rHDL stimulated faster and more efflux than did the free protein (data not shown), consistent with previous results (35). No significant differences were detected between plasma-derived human apoA-I, T7-hAI, or T7-mAI in the lipid-free or lipid-bound state. In view of these findings, the cholesterol efflux induced by the chimeric apoA-Is was not assessed.

Association Assay—By having established that the recombinant wild-type human apoA-I is functionally and structurally similar to the plasma protein, we proceeded to examine the ability of the various recombinant proteins to associate with mature HDL subclasses. In the association assay, lipid-free recombinant apoA-Is were incubated with TLP from human plasma (36) at an endogenous to recombinant apoA-I ratio of 62:5:1. At this ratio the added apoA-I “tags” the HDL without significantly altering the HDL size or density. Assuming three molecules of apoA-I per HDL, there are about 20 HDL particles (HDL2 + HDL3) per recombinant apoA-I molecule in the association assays. Since there is an excess of HDL, the recombinant apoA-I binds to that HDL subclass exhibiting the highest affinity for the apoA-I.

The HDL subclasses were separated after incubation with the recombinant apoA-Is on NaBr equilibrium density gradients, and the fractions were analyzed for the presence of endogenous apoA-I and recombinant proteins by immunoblotting. Fig. 5A shows a tracing of the protein distribution across the lipoprotein profile (A260). Endogenous apoA-I was detected with HDL lipoproteins (Fig. 5B) but not VLDL/LDL lipoproteins (data not shown). In addition, ~3% of endogenous apoA-I was found in the free protein fractions. This was likely loosely associated apoA-I that was stripped from the lipoprotein particles during ultracentrifugation (37). The starting protein ratios of HDL2:HDL3 for the six association experiments ranged between 45:55 and 60:40, as determined by measuring the area under the curve of the lipoprotein tracings at A280. In order to compare data among experiments, the recombinant apoA-I distributions were normalized to the starting HDL2:HDL3 proportions by dividing the recombinant apoA-I distributions by the corresponding endogenous apoA-I distributions ratios. Endogenous apoA-I is an appropriate surrogate for starting HDL subclass proportions, as demonstrated by the similar HDL subclass ratio calculated from A260 tracings and from the densitometric scanning of the Western blot for endogenous apoA-I (Fig. 5C).

T7-hAI distributed to the HDL subclasses in a pattern that followed closely that of endogenous apoA-I (Figs. 5B and 6). On average, 50% of T7-hAI distributed to HDL2 and 50% to HDL3, showing that T7-hAI bound with approximately equal affinity to HDL2 and HDL3. Of the T7-mAI that bound to HDL, 66% distributed to HDL2 and 34% to HDL3, showing that T7-mAI bound preferentially to HDL2 compared with HDL3 (Figs. 5B and 6). In addition, more T7-mAI was found in the free fractions than the human apoA-I proteins.

The chimeric recombinants were then examined in the same assay (Figs. 5B and 6). With the chimera T7-hAI(m7/8), 62% of the added protein was found in the HDL3 range and 38% in HDL2. Although the majority of the sequence of this chimera was derived from human apoA-I, the association profile more closely resembles that of T7-mAI than T7-hAI. Conversely, 45 and 55% of T7-mAI(h7/8) distributed to HDL2 and HDL3, re-
intermediate density lipoprotein, HDL2, and, to a small extent, of the HDL in peak HDL2 and HDL3 fractions from TLP incubation with LDL fractions (38, 39).

The ratios of HDL:recombinant apoA-I ranged from throughout the HDL fractions, apoA-II distributing predominantly in the HDL subclasses, and apoE distributing to VLDL/intermediate density lipoprotein, HDL2, and, to a small extent, in the LDL fractions (38, 39).

The effect of recombinant apoA-I on HDL size was also monitored as a second assessment of HDL remodeling. The size of the HDL in peak HDL2 and HDL3 fractions from TLP incubated with buffer, T7-hAI, or T7-mAI was determined along with control HDL2 and HDL3, which had Stokes diameters of 10.2 and 8.4 nm, respectively. These diameters did not change when HDL was incubated with recombinant apoA-I (data not shown).

**HDL Remodeling in Association Assays**—With the low levels of recombinant apoA-I added to TLP, minimal HDL remodeling was anticipated. However, to check for HDL remodeling, endogenous apoA-I, apoA-II, or apoE distributions were determined. Incubation with T7-hAI or T7-mAI did not affect any of the endogenous apoprotein distribution profiles nor was the extent of endogenous apoproteins found in the free protein fractions influenced (data not shown). In all cases the apoprotein distributions were as expected, with apoA-I distributed throughout the HDL fractions, apoA-II distributing predominantly in the HDL2 fractions, and apoE distributing to VLDL/intermediate density lipoprotein, HDL2, and, to a small extent, in the LDL fractions (38, 39).

**Binding Curves of Recombinant ApoA-I to HDL**—In the above association assays, a small amount of recombinant apoA-I was incubated with a mixture of HDL2 and HDL3 so that these two HDL subclasses competed for the added recombinant apoA-I. The results from these experiments suggested that the affinity of some of these recombinants differ for the two HDL subclasses. Since added apoA-I did not cause remodeling of HDL or significant desorption of endogenous apoA-I, the binding curves of apoA-I for HDL subclasses could be measured directly using Scatchard analysis. This analysis was based on reversible binding of apoA-I to site on an HDL particle as shown in Equation 1,

$$\text{apoA-I+site} \rightarrow \text{complex} \quad K_d \quad \text{(Eq. 1)}$$

Since (Site)$_{\text{free}} = (\text{Sites})_{\text{total}} - (\text{Sites})_{\text{occupied}} = (\text{Sites})_{\text{total}} - (\text{apoA-I$_{\text{bound}}$})$ and $C_x = (\text{apoA-I$_{\text{bound}}$})$, the value of $K_d$ and (Site)$_{\text{total}}$ could be assessed as parameters using non-linear least square analysis.

For determination of these binding curves, 10, 20, 40, or 80 μg of recombinant apoA-I was incubated with 200 μg of isolated HDL2 or HDL3, corresponding to 130 or 150 μg of endogenous apoA-I. The ratios of HDL:recombinant apoA-I ranged from 3.2:1 to 0.4:1 for HDL2 (assuming 4 apoA-Is/HDL2) and from 5:1 to 0.6:1 for HDL3 (assuming 3 apoA-Is/HDL3). Scatchard analysis (Fig. 7) demonstrated that T7-hAI binding to HDL2 and HDL3 and T7-mAI binding to HDL2 was specific. For the binding of T7-mAI to HDL3, the $K_d$ was essentially infinite suggesting T7-mAI binding to HDL3 was of very low affinity. In Fig. 7, we have not plotted concentrations above 40 μg (2.3 μM) for T7-hAI (A and B) or above 50–70 μg (2.9–4 μM) for T7-mAI (C and D) because at these high concentrations there is evidence of remodeling of HDL particles (see below). The lower $K_d$ values of T7-hAI for both HDL2 and HDL3 (1.52 and 2.41 μM, respectively) than of T7-mAI for HDL2 (6.28 μM) demonstrate that T7-hAI has a higher affinity for HDL than does T7-mAI. The $B_{\text{max}}$ (i.e., the apoprotein concentration at which the available sites on HDL are filled without remodeling) for T7-hAI with HDL2 and HDL3 was 9.5 and 10.7 μg, respectively. A $B_{\text{max}}$ of 9.9 μg was obtained for T7-mAI with HDL2. These findings indicated that recombinant apoA-I saturated HDL2 at similar levels.

**Total HDL and HDL2/HDL3 Ratios of Recombinant ApoA-I at 40 μg**—In order to compare the binding of the various types of recombinant apoA-Is to HDL, two values were calculated from the binding curves. For these calculations the value of 40 μg of added apoA-I per HDL subclass was chosen because it was the highest where no HDL remodeling was evident (see below). The first value is the sum of the amounts of recombinant apoA-I bound to HDL2 plus HDL3 (total HDL association). The second value is the ratio of recombinant apoA-I bound to HDL2 or HDL3 (the HDL2/HDL3 ratio). The amount of recombinant apoA-I bound to total HDL was more for T7-hAI (42.2 μg) than T7-mAI (23.4 μg), consistent with either a higher binding affinity of T7-hAI compared with T7-mAI or a higher $B_{\text{max}}$ of T7-hAI as compared with T7-mAI. Since the $B_{\text{max}}$ of the two proteins were found to be similar, it is likely that T7-hAI has a higher HDL affinity than T7-mAI. The HDL2/HDL3 ratio was 1.3:1 for T7-hAI and 1.8:1 for T7-mAI. These ratios suggest that T7-mAI has a higher affinity for HDL3 than HDL2, whereas T7-hAI has similar affinities for HDL2 and HDL3.

Next, the total HDL association values were determined for

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**Table II**

**Characterization of recombinant apoA-Is and human plasma apoA-I in rHDL and lipid-free forms**

| Protein component | POPC:FCapoAI molar ratio | rHDL diameter | ApoA-I/rHDL | α-Helix content |
|-------------------|--------------------------|---------------|-------------|---------------|
|                   | Initial | Final | NDGGE | EM | Lipid-free | rHDL/ |
|                   | | | nm | nm | % | % |
| Human apoA-I | 80:4:1 | 78:4:6:1 | 9.8 | 10.0 ± 1.5 | 2 | 40 ± 8.7 | 68 |
| T7-hAI | 80:4:1 | 70:4:1:1 | 10.2 | 9.9 ± 1.3 | 2 | 38 ± 4.4 | 63 |
| T7-mAI | 80:4:1 | 73:5:3:1 | 10.2 | 9.8 ± 1.5 | 2 | 32 ± 2.2 | 61 |
| T7-hAI(m7/8) | 80:4:1 | 70:4:0:1 | 10.3 | 9.8 ± 1.5 | 2 | 35 ± 5 | 65 |
| T7-mAI(h7/8) | 80:4:1 | 75:3:4:1 | 10.3 | 9.9 ± 1.1 | 2 | 33 ± 4 | 60 |

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**Table III**

**LCAT activation by recombinant apoA-Is**

| ApoA-I construct | Maximal LCAT activity |
|------------------|-----------------------|
| Plasma apoA-I | 100 |
| T7-hAI | 79 ± 1.0* |
| T7-mAI | 79 ± 1.0* |
| T7-hAI(m7/8) | 105 ± 8.0 |
| T7-mAI(h7/8) | 74 ± 10.0* |

*$p < 0.005$.

*$p < 0.05$, compared with plasma apoA-I.
the chimeric recombinants \((n = 2)\). The T7-hAI(m7/8) had a total HDL value of 49.6 \(\mu\)g, similar to T7-hAI. Conversely, T7-mAI(h7/8) had a total HDL value of 20.3 \(\mu\)g, similar to T7-mAI. Thus, the amount of the chimera that binds to HDL is similar to that of the parent apoA-I, suggesting that helices 7/8 do not play a major role in determining total HDL binding. These findings are consistent with the studies that show that helices 1, 9, and 10 of human apoA-I are essential for the high lipid binding affinity of human apoA-I (10, 31, 32). Finally, the HDL2:HDL3 ratio for the chimeras. This ratio for T7-hAI(m7/8) was 2.0:1, similar to the value obtained with T7-mAI (1.8:1). The only difference between the two proteins is in the source of helices 7/8 that is expressed, suggesting that they contribute to the association of apoA-I with HDL subclasses. Correspondingly, the HDL2: HDL3 ratio for T7-mAI(h7/8) was 1.5:1, which is similar to the value obtained for T7-hAI (1.3:1).

**HDL Remodeling in Binding Assays**—When 20, 40, or 80 \(\mu\)g of T7-hAI or T7-mAI was incubated with either HDL subclass in the binding assays, the Stokes diameters did not change (data not shown), indicating that by this parameter major HDL remodeling did not occur. However, when displacement of endogenous proteins was assessed, the addition of >40 \(\mu\)g of T7-hAI or T7-mAI resulted in significant displacement of apoA-I from both HDL subclasses. We consistently observed more endogenous apoA-I in the free protein fractions when higher levels of T7-hAI were added than when similar amounts of T7-mAI were added. These findings suggest that with the addition of recombinant apoA-I there is competition with endogenous apoA-I for the HDL surface, displacing some of the latter. This competition is more effective with T7-hAI, which binds to HDL with higher affinity than T7-mAI and therefore is more likely to displace endogenous apoA-I.

**DISCUSSION**

Recombinant human, mouse, and chimeric apoA-I proteins were generated to determine domains of apoA-I that may account for the distinct human and mouse HDL profiles. Our results provide evidence for a strong participatory role of helices 7/8 in determining the selective patterns of HDL association.

In order to carry out these studies, N-terminal epitope-tagged apoA-Is were made as bacterially expressed recombinants. The T7 epitope allowed for distinguishing exogenous apoA-I on immunoblots. The His\(_6\) tag was included to facilitate the purification of the recombinant molecules. To assure ourselves that these tags did not perturb the properties of the resultant apoA-I molecules, we examined several physical and functional properties of these recombinants. The properties studied included molecular size, proportion of \(\alpha\)-helix in the lipid-free state or in the presence of lipid, self-association properties of the free protein, and the capacity to promote cholesterol efflux from cholesterol-labeled fibroblasts. The molecular weights of the recombinants were confirmed by electron spray mass spectrometry. There were no differences in the proportion of \(\alpha\)-helix or the capacity to promote cholesterol efflux among each of the recombinants. However, there were informative differences in the self-association properties and in the capacity to activate LCAT.

Human apoA-I is well known to form oligomers in the absence of lipid. A previous study (30) has suggested that mouse
apoA-I oligomerizes to a lesser extent than human apoA-I; our results support this finding (Fig. 3). Notably, even when human helices 7/8 were incorporated into the mouse backbone, the protein self-associated as though it were a mouse protein. Similarly, the incorporation of mouse helices 7/8 had little or no effect on the self-association properties of the human protein. These data clearly indicate the helices 7/8 do not govern the distinct self-association properties of human and mouse apoA-I. Comparison of human and mouse hydropathy plots as well as analysis of the properties of C-terminal deletion mutants of human apoA-I (10, 31, 32) suggest that helix 10 or helices 9/10 confer the distinct self-association profiles.

Mouse apoA-I does not activate human LCAT as effectively as human apoA-I (Table III), similar to previous reports (34). The exchange of helices 7/8 did not influence the relative activation capacities of human and mouse apoA-I. Thus, the incorporation of murine helices 7/8 into the human backbone still produced a highly efficient activator of human LCAT. The reciprocal chimera behaved like a mouse apoprotein with respect to LCAT activation. These data argue that differences between human and murine helices 7/8 do not play a major role in the effectiveness with which apoA-I activates LCAT and are consistent with the importance of helix 6 in LCAT activation (41).

We have performed two related assays to ascertain how murine and human apoA-I associate with HDL of different size and radius of curvature. In the first assay, which we have designated the association assay, a low concentration of recombinant apoA-I is incubated at 4 °C with total human lipoprotein containing HDL$_2$ and HDL$_3$. The recombinant protein partitions between the two HDL species. In initial experiments using human lipoproteins, the HDL subclass distribution of His$_6$-hAI (generous gift of Dr. Yves Marcel) and T7-hAI were similar (data not shown), indicating that the T7 epitope tag does not interfere with these patterns. Human lipoprotein was employed as the recipient for these associations because it contains both major HDL species. When mouse lipoprotein was used as the recipient, no distinction was noted between human and mouse apoA-I (data not shown). In view of their similar association with HDL$_2$, this is an expected outcome. In this association assay, T7-hAI distributes in direct proportion to the endogenous apoA-I. On the other hand, murine apoA-I shows a clear preference for HDL$_2$. This same preference is exhibited by human apoA-I containing the mouse helices 7/8. The mouse protein containing human helices 7/8 distributes to both HDL subclasses equally. This assay is similar to that previously employed by Dong and Weisgraber (42) in which they revealed the preference of apoE4 for VLDL in contrast to apoE3 which had a preference for HDL. It is also inherent in the HDL turnover studies that have been performed after incubation of exogenously labeled apoA-I with HDL prior to injection into rabbits (43).

In the second assay, we determined binding curves of the recombinant apoproteins with isolated HDL$_2$ and HDL$_3$ individually so there was no competition between HDL$_2$ and HDL$_3$ for apoA-I. From this assay several parameters could be calcu-
lated as follows: an apparent \( K_d \) for mouse and human apoA-I for HDL2 and HDL3, an apparent site total for this binding; a ratio of HDL2 to HDL3 binding using the 40-μg amount of added apoprotein per HDL subclass; and finally, the amount of apoA-I bound to HDL2 plus that bound to HDL3 at the 40-μg concentration per HDL subclass. The apparent \( K_d \) value for T7-hAI binding to HDL2 or HDL3 was similar, as was the \( K_{H1} \) for T7-mAI, the apparent \( K_d \) value for HDL3 was 2–4-fold higher than the T7-hAI \( K_d \) value for HDL2 and HDL3. The T7-mAI \( K_d \) value for HDL3 could not be calculated since there was no evidence of saturation, indicating low affinity of T7-mAI for HDL3. All of these findings reinforce the results of the first assay as follows: 1) T7-hAI binds equally well to HDL2 and HDL3; 2) T7-hAI has a binding affinity for both HDL subclasses that is greater than that of T7-mAI; and 3) T7-mAI has a relatively higher affinity for HDL3 than HDL2.

Interestingly, the affinities for preformed HDL subclasses, as shown in the association assays and the binding curves, parallel the size and density of HDL that human and mouse apoA-I form in their respective plasmas. This suggests that the factors leading to the distinct associations in vitro may contribute to HDL formation in vivo. This is consistent with the finding from apoA-I transgenic mice, in which the mice develop a human-like HDL profile (3). Our in vitro studies are also interesting in that they demonstrate that the HDL affinity of human apoA-I is greater than mouse apoA-I. It may be hypothesized that this is the basis for the findings that murine apoA-I plasma levels are almost non-existent in human apoA-I transgenic mice, despite normal liver mRNA levels (3).

We have complete data for the binding of the chimeras to the HDL subclasses at the 40-μg level of added apoprotein per HDL subclass. The total HDL binding of T7-hAI and the T7-hAI/m7/8 chimera was higher (42–50 μg bound) than that for T7-mAI and the T7-mAI/h7/8 chimera (20.3–23.4 μg bound). These data suggest that T7-hAI has a higher affinity for HDL and that the origin of helices 7/8 does not affect this affinity. Prior studies (1, 32, 33) have suggested that helices 1, 9, and 10 are the helices thought to be involved in lipid binding, and these differences could account for the self-association properties of the proteins we have observed. Within the individual helices, the overall distribution of charged and hydrophobic residues is generally maintained between the mouse and human proteins. However, helices 7/8 exhibit a large sequence diversion (~50%), and these differences appear to relate strongly to the ability of the respective proteins to associate with different HDL subclasses. We presume that this relates to the ability of the proteins to associate with HDL particles with different radii of curvature, with the mouse protein not as readily able to accommodate the higher radius of curvature of HDL3. Based upon the prior definition of the hinge domain of apoA-I (helices 4–7), it is not surprising that these two helices should play a significant role in the targeting of various apoA-1s to HDLs of differing size and radius of curvature. Of particular interest here are the differences in the boundaries between helices 7 and 8 in human and mouse apoA-I. In the human apoprotein this region contains two glycine residues and lacks the typical proline punctuation found at most of the other inter-helical boundaries. However, the mouse apoprotein does have the proline residue in this position. In addition, in the mouse protein these two glycines are deleted resulting in a mouse helix 7 that contains only 20 amino acids rather than the canonical 22 amino acids characteristic of most of the helical repeats in apoA-I. The fact that helix 7 in the mouse is two residues shorter or the absence of the two glycine residues in the interhelical region in the mouse may result in conformational changes in the protein C-terminal to helix 7 that alter its ability to associate with HDL of different radii of curvature (4).

The designation of the helical repeats in apoA-I is based largely on the presence of the 11/22-mer repeat sequences in the protein that can form amphipathic α-helices in vitro (44). The boundary between these putative helices is most often punctuated by a proline residue. In the low resolution crystal structure of residues 44–243 of human apoA-I, most of this region is helical, with the exception of the beginning and end of the N-terminal truncated protein (45). In this crystal structure, residues 165–208 form a continuous α-helix with only a 12° turn between the putative helices 7 and 8. In contrast, a sharp bend occurs in the preceding four interhelical regions and the helix 9/10 interhelical region. Each of these interhelical regions contains a proline residue. The presence of the proline residue in mouse apoA-I in the 7/8 interhelical turn may introduce a sharp bend in the structure of the mouse protein at this point. In the human protein, the 7/8 interhelical turn appears to be located in a loop/disordered region. This is based on the increased thermal and chemical stability of lipid-free apoA-I in which proline residues are substituted for the glycine residues at positions 185 and 186 in the interhelical turn (46). The introduction of the prolines may provide more restrictions on the conformation of that area. The long continuous helix 7/8 and the disorder structure in the putative interhelical turn is consistent with this region of human apoA-I being more flexible to tolerate HDL particles with different radii of curvature, while in the mouse this region is more constrained due to the presence of the proline residue, thus limiting its ability to adjust to differences in the size of the HDL particles.

The functional basis for the development of HDL heterogeneity in primates is not clear. The ability to study this depends upon the capacity to generate HDLapoA-I or HDLapoA-I only animals based upon the human or mouse sequences of apoA-I. The studies reported here represent a start in the generation of such animals, for the defined exploration of the functional significance of HDLapoA-I and HDLapoA-I in general, and in the protection against the development of atherosclerosis in particular.

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