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Characterization of a Family of Gamma-Ray-Induced CHO Mutants Demonstrates that the *ldlA* Locus Is Diploid and Encodes the Low-Density Lipoprotein Receptor

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The *ldlA* locus is one of four Chinese hamster ovary (CHO) cell loci which are known to be required for the synthesis of functional low-density lipoprotein (LDL) receptors. Previous studies have suggested that the *ldlA* locus is diploid and encodes the LDL receptor. To confirm this assignment, we have isolated a partial genomic clone of the Chinese hamster LDL receptor gene and used this and other nucleic acid and antibody probes to study a family of *ldlA* mutants isolated after gamma-irradiation. Our analysis suggests that there are two LDL receptor alleles in wild-type CHO cells. Each of the three mutants isolated after gamma-irradiation had detectable deletions affecting one of the two LDL receptor alleles. One of the mutants also had a disruption of the remaining allele, resulting in the synthesis of an abnormal receptor precursor which was not subject to Golgi-associated posttranslational glycoprotein processing. The correlation of changes in the expression, structure, and function of LDL receptors with deletions in the LDL receptor genes in these mutants directly demonstrated that the *ldlA* locus in CHO cells is diploid and encodes the LDL receptor. In addition, our analysis suggests that CHO cells in culture may contain a partial LDL receptor pseudogene.

Low-density lipoprotein (LDL), the principal cholesterol transporter in human plasma, is taken up by cells via receptor-mediated endocytosis (11). Much of our understanding of the receptor-mediated endocytosis of LDL has come from comparisons of normal human cells and mutant cells derived from patients with familial hypercholesterolemia. This disease, which results in premature atherosclerosis, is a consequence of mutations in the structural gene for the LDL receptor (12). To extend the genetic analysis of LDL endocytosis, we have isolated a collection of Chinese hamster ovary (CHO) cell mutants which cannot bind or internalize LDL (26, 28). These LDL receptor-deficient mutant cells belong to four distinct genetic complementation groups: *ldlA*, *ldlB*, *ldlC*, and *ldlD* (21, 27).

The LDL receptor deficiencies in *ldlB*, *ldlC*, and *ldlD* mutants are consequences of mutations which change the structures of many cellular glycolipids and glycoproteins, including the LDL receptor (20). The source of these abnormalities in *ldlD* cells is a deficiency in UDP galactose/UDP-*N*-acetylgalactosamine 4-epimerase activity, which affects the production of nucleotide sugars required for the glycosylation of proteins and lipids (19). The specific defects in *ldlB* and *ldlC* mutants have not yet been elucidated; however, they appear to affect multiple activities associated with the Golgi complex (20, 22).

The *ldlA* locus has been tentatively identified as the structural gene for the LDL receptor in CHO cells, based on several lines of evidence. (i) Unlike mutants from the other three complementation groups, *ldlA* mutants could not be complemented by fibroblasts isolated from patients with familial hypercholesterolemia (21). Since these patients have mutations in the LDL receptor gene (12), this result suggests that the *ldlA* mutants also had defects in the LDL receptor gene. (ii) In gene transfer studies, the receptor-negative phenotype of an *ldlA* mutant was specifically corrected by the introduction and expression of the human LDL receptor gene (40). A different human gene corrected the multiple defects in an *ldlB* mutant (22). (iii) Multiple mutant forms of the receptor have been detected in *ldlA* mutants (24).

In the current work, we have constructed a family of mutants to characterize the *ldlA* locus directly. Since gamma irradiation of CHO cells often induces substantial genetic deletions or chromosomal rearrangements (14, 47, 50, 52), we have isolated a family of *ldlA* mutants after gamma irradiation of wild-type and revertant cells and determined the relationship between changes in the structure and expression of LDL receptors with changes in the structure of the LDL receptor gene.

MATERIALS AND METHODS

DNA size standards, cloning vectors, oligomers, restriction enzymes, and polymerases were obtained from New England BioLabs, Inc., Amersham Corp., Promega Biotech, International Biotechnologies, or Boehringer Mannheim Biochemicals. Random hexanucleotides and dextran sulfate were obtained from Pharmacia. [α-thio-^{35}S]dCTP (>600 Ci/mmol) used in sequencing was obtained from Amersham. [^{35}S]methionine (>800 Ci/mmol) and [^{32}P]dCTP (>3,000 Ci/mmol) were purchased from New England Nuclear Corp. DNA isolated from the V20.2 strain of the V-79 Chinese hamster cell line was generously provided by Phillipe Gros and David Housman. LDL and lipoprotein-deficient serum were prepared by ultracentrifugation (25). All other immunological (24), biochemical (21, 40) and tissue culture (25) reagents were obtained as previously described or from Sigma Chemical Co.

Tissue culture. Wild-type and mutant CHO cells were grown as previously described (24) except that stock cultures of CHO cells were grown in medium supplemented with 5% (vol/vol) fetal bovine serum rather than 10% (vol/vol) newborn bovine serum. ldlA-7 refers to mutant clone 7 (21) and RevA7 refers to RevA7-1, a spontaneous

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revertant derived from ldlA-7 (21). ldlA-650 cells were isolated by MeLoCo-amphotericin B selection (21, 28) of CHO cells which had been subjected to gamma irradiation (667 rads). This mutant clone was the only colony isolated from 5×10^6 irradiated cells subjected to selection, a frequency much lower than the frequency at which *ldlA* mutants arise after ethyl methanesulfonate (EMS) mutagenesis (1 to 25 colonies per 10^6 cells [21]). We have never isolated an *ldlA* mutant directly from nonmutagenized CHO cells (frequency, <1 colony per 10^7 cells, [21]). Gamma-irradiated mutant RA7-64 (γRA7-64) and γRA7-89 were independent colonies isolated from independently gamma-irradiated (600 rads) stocks of RevA7 cells by the same selection technique (10^6 cells of each independently irradiated stock of RevA7 cells were subjected to two rounds of MeLoCo-amphotericin B selection [28]). The average frequency at which receptor-negative mutants were isolated from five independently irradiated stocks (10^6 cells per stock) was 22 colonies per 10^6 cells. γRA7-64, γRA7-89, and ldlA-650 were assigned to the *ldlA* complementation group (21; data not shown).

Bacterial culture and molecular cloning. Most bacterial culture and recombinant DNA techniques were performed by using standard techniques (17, 33, 34). Restriction fragments for subcloning were purified from low-gel-temperature agarose (Sea-Plaque; FMC Corp., Marine Colloids Div.) by using hexadecyl trimethylammonium bromide (29). A genomic cosmid library prepared from an *Msp*I partial digest of DNA from a Chinese hamster V-79 cell line (15) was generously provided by P. Gros and D. Housman, Massachusetts Institute of Technology.

Cosmid clone *cos*5A7-1-1 was isolated (16) from this library on the basis of hybridization to probes (Bov1518 and Bov1214 [see below]) prepared from the cloned bovine LDL receptor cDNA (37, 38). Because a portion of *cos*5A7-1-1 is homologous with Bov1518, this cosmid contains a portion of the hamster LDL receptor gene, not the apparent pseudogene described in this study (see Results and Discussion). pV79-1 was a 1.2-kilobase (kb) *Xba*I fragment of this cosmid, cloned into vector pUC18 (54). Ham12 was an approximately 600-base-pair *Xba*I-*Ban*II repeat-free fragment of this subclone.

Isolation of other DNA probes. Plasmids pLDLR1, pHH1, and pLDLR3 were gifts of D. Russell, M. Brown, and J. Goldstein, Dallas, Tex. Bov1518 (base 418 to an *Xba*I site at about base 1000) and Bov1214 (bases 1 to 417) were isolated from the bovine partial LDL receptor cDNA clone, pLDLR1 (37, 38). Hum1-4 and Hum8-10 were derived from human LDL receptor cDNA clone pLDLR3 (similar to pLDLR2 [53; D. Russell, personal communication]). Hum1314 and Hum1517 were subclones of plasmid pHH1 (a partial human cDNA clone including bases 1573 to 3486 [53; D. Russell, personal communication]). The location of each probe used in this study, relative to the human cDNA sequence (53), is listed in Table 1.

Southern blots. Southern blot analysis was performed as described previously (43, 51) except that HCl treatment of the gel was omitted and nylon membranes (Zetabind; AMF Cuno) were substituted for nitrocellulose or diazobenzyloxymethyl-paper. ^{32}P-labeled *Hin*dIII-digested lambda DNA (New England BioLabs) was used as a molecular weight standard. After digestion with an excess of restriction enzyme, DNA concentration for gel loading was estimated by a fluorometric assay (18). Hybridization probes were labeled to a specific activity of 10^8 to 10^9 cpm/μg by hexanucleotide-primed DNA synthesis (9, 10) in the presence of [^{32}P]dCTP. After hybridization, blots were washed with either 0.2× SSPE (1× SSPE contains 180 mM NaCl, 10 mM $Na_2H_2PO_4$, and 1 mM EDTA, [pH 7.4])–0.1% (wt/vol) sodium dodecyl sulfate at 65°C for pER7.8-probed Southern blots or 1.2× SSPE–0.1% sodium dodecyl sulfate at 55°C for other blots. Autoradiograms were exposed for 12 to 120 h at −80°C with presensitized Kodak XAR-5 film in the presence or absence of an intensifying screen (Cronex Lightning-Plus; E.I. du Pont de Nemours & Co., Inc. [30]). Relative band intensities were determined by averaging the integrated intensities of four laser densitometer (model 2202; LKB Instruments, Inc.) track recordings of each lane (average standard deviation, 15%). To correct for differences in the amount of DNA loaded in each lane, we divided the intensity of each band by the intensity of a single 7.8-kb band observed in the same lane when the filter was probed with an unrelated single-copy Chinese hamster gene fragment, pER7.8. This control probe (pER7.8) is a fragment of a Chinese hamster multidrug resistance gene (15), provided by P. Gros and D. Housman, Massachusetts Institute of Technology. Use of an intensifying screen had no effect on the relative intensities (data not shown). The reliability and reproducibility of the data presented here were verified in a number of control experiments (data not shown), which included analysis of duplicate samples on the same filter, analysis of independently prepared filters and probes, and measurement of the intensities of bands from different autoradiographic exposures of the same blot.

Sequence analysis. The M13MP8 and M13MP9 subclones used for sequencing were derived from a Ham12-containing pGEM-1 (Promega Biotech) clone. Deletion subclones were obtained by using the single-stranded T4 DNA polymerase deletion technique (5). *Escherichia coli* JM103 was used for the transformation and growth of all of the M13 constructs. DNA-sequencing reactions were carried out by the dideoxy chain termination method (39) with [α-thio-^{35}S]dCTP. The sequence reported here was determined from the analysis of two overlapping subclones from each strand. DNA sequence data were analyzed by using standard computer programs (7, 8, 35, 44, 45) and the National Biomedical Research Foundation nucleic acid sequence database, release 25 (1). The base-numbering schemes reported in the original studies (37, 53) were used.

Immunoprecipitation. Metabolic labeling of cells with [^{35}S]methionine, treatment of cells with tunicamycin or pronase, endoglycosidase H treatment of cell extracts, immunoprecipitation with anti-C or anti-R antibodies, electrophoresis, and autoradiography were performed as previously described (24). Incubation of cells in the presence of exogenous sterols was performed as previously described (40). Membrane and soluble fractions of homogenized cells were prepared by ultracentrifugation (24).

RESULTS

Sequence of a Chinese hamster LDL receptor DNA probe. To further analyze the nature of the genetic defects in our collection of *ldlA* mutants, we cloned a fragment of the Chinese hamster LDL receptor gene. A portion of this clone (Ham12) was sequenced and found to be highly homologous to exon 12 of the human LDL receptor (Fig. 1) (48, 53). In the hamster, human (53), and bovine (37) LDL receptor sequences, 82% (89/108) of the nucleotides and 86% (31/36) of the predicted amino acids within the putative coding region of the fragment are identical. The Chinese hamster sequence contained consensus splice sites at positions

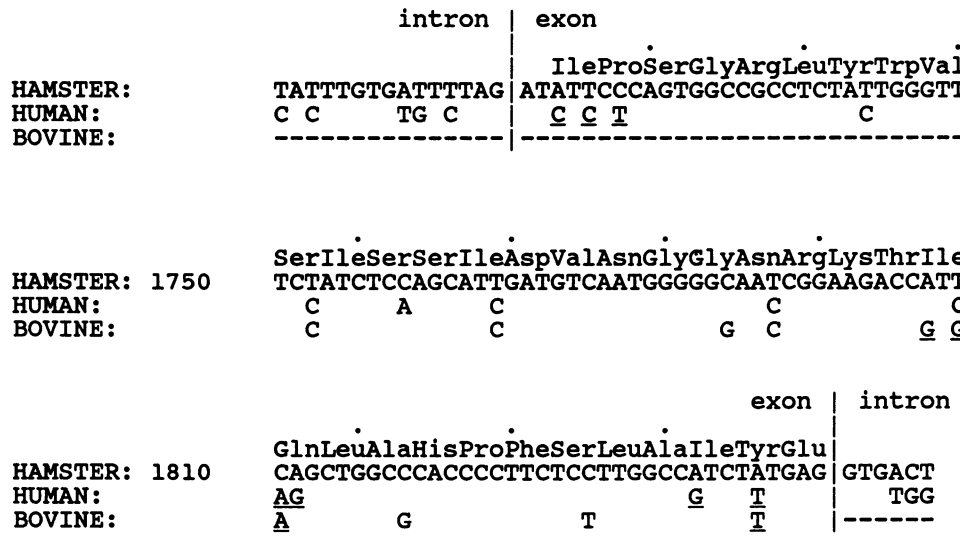

FIG. 1. Sequence comparison of hamster, human, and bovine LDL receptor genes. The Chinese hamster sequence was obtained as described in the text. The amino acid sequence shown was deduced from this DNA sequence. The human DNA sequence was taken from the cDNA sequence of exon 12 (bases 1706 through 1861 [53]) and its flanking genomic sequence (intron [48]). Base numbers used refer to the human cDNA sequence (53). The bovine sequence was obtained from a cDNA clone (37) beginning at base 1738. Only the bovine and human nucleotides which differ from those of the hamster sequence are indicated. Those nucleotides which result in altered amino acids are underlined. Intron and exon designations refer to the human gene sequence and coincide with consensus splice sites (3, 44) in the Chinese hamster sequence. Dashed lines indicate unavailable data.

equivalent to those which define human exon 12, suggesting that these intron-exon boundaries were conserved (Fig. 1). The sequence of the Chinese hamster LDL receptor gene beyond the putative 5′ splice donor site contains an in-frame stop codon (13 bases past the splice site; data not shown) and fails to show the positional base preference which is found in many DNA sequences which encode proteins, including the portion of Ham12 which is homologous with human exon 12 (44; data not shown). As is the case with the human (48, 53) and bovine (37) LDL receptor genes, the amino acid sequence predicted by this fragment (Fig. 1) was homologous with a portion of the murine epidermal growth factor precursor gene (z score, 8.0 standard deviations [7]).

In addition to Ham12, we have used portions of the human and bovine cDNAs to analyze CHO mutants. The positions of these hamster (Ham12), human (Hum1-4, Hum8-10, Hum1314, and Hum1517), and bovine (Bov1518 and Bov1214) DNA probes, relative to the human LDL receptor gene map, are summarized in Table 1.

TABLE 1. DNA probes

Probe	Source (reference)	Corresponding human exons[a]	Corresponding human bases[b]
Hum1-4	Human cDNA (53)	1–4	−13–718
Hum8-10	Human cDNA (53)	8–10	1077–1448
Ham12	Chinese hamster genome	12	1706–1845[c]
Bov1214	Bovine cDNA (37)	12–14	1738–2155[d]
Hum1314	Human cDNA (53)	13–14	1866–2154
Hum1517	Human cDNA (53)	15–17	2155–2546
Bov1518	Bovine cDNA (37)	15–18	2155–2580[e]

[a] The human LDL receptor has 18 exons which have been numbered beginning at the 5′ end of the cDNA sequence (48). Each probe contains or is homologous to at least 25 bases in each exon of the indicated range.

[b] Human base numbers were taken from reference 53.

[c] This probe also contains flanking Chinese hamster intron sequences.

[d] Homologous bases were determined by comparing portions of the human and bovine LDL receptor cDNA sequences by using the GAP program (8) with a gap penalty of 5.00 and a length weight of 0.5.

[e] Base 2580 is the last base of the coding region of the human LDL receptor cDNA. Both human and bovine cDNAs contain extensive 3′ untranslated regions. Bov1518 contains about 250 bases beyond the end of the coding region.

Isolation and characterization of *ldlA* mutants isolated after gamma irradiation. We have isolated and characterized several *ldlA* mutants after gamma irradiation (Fig. 2). Mutant cells ldlA-7 (21) and ldlA-650 were isolated from wild-type cells after EMS and gamma-ray mutagenesis, respectively. Both mutants were receptor negative (<1% of wild-type receptor activity). RevA7 revertants, which were isolated by MeLoCo selection (21, 40), arose spontaneously from ldlA-7 cells and had approximately 50% of the wild-type LDL receptor activity (21). γRA7-64 and γRA7-89 are two independent receptor-negative (<1% of wild-type receptor activity) mutants which were isolated by MeLoCo-amphotericin B selection (28) of gamma-irradiated RevA7 cells.

These cells were pulse-labeled with [^{35}S]methionine for 30 min, followed by a 30-min chase (Fig. 3). The LDL receptors were then immunoprecipitated and analyzed by gel electrophoresis and autoradiography. LDL receptors were immunoprecipitated with anti-C, an antibody directed against the C terminus of the LDL receptor (23) (Fig. 3, lanes 1 through 6). Wild-type cells (lane 1) synthesized a precursor form of the LDL receptor (Fig. 3, p; mass, 125 kilodaltons [kDa]), which was almost entirely converted to the mature form (m; mass, 155 kDa) during the chase. In contrast, the precursors synthesized by ldlA-7 cells (mass, 125 kDa; Fig. 3, lane 2) were not processed during this short chase. During longer chases, the LDL receptor precursors produced by this mutant are slowly and continually processed to increasingly higher molecular weight intermediates (125 to 135 kDa [24]). These abnormal intermediate forms are rapidly degraded and do not reach the cell surface (24). RevA7 cells synthesized receptors with both ldlA-7-type and wild-type mobilities (Fig. 3, lane 3) (24). The presence of two types of LDL receptors in the RevA7 cells strongly suggests that the *ldlA* locus is diploid and encodes the LDL receptor.

Only an abnormal form of the receptor was detected in the γRA7-64 (Fig. 3, lane 4) and γRA7-89 (lane 5) cells. The abnormal processing of the LDL receptor precursor in these cells was essentially identical to that of the abnormal receptors in the ldlA-7 and RevA7 cells (data not shown). Thus, it appears that the receptor-negative phenotype of the γRA7 cells arose as a consequence of the inactivation of the single, functional LDL receptor allele in the RevA7 cells (see below) after gamma-ray mutagenesis. Anti-C detected no specifically immunoprecipitable material in the ldlA-650 cells (Fig. 3, lane 6). None of the receptor proteins described above were detected by using preimmune immunoglobulin G, whereas all of the background bands seen in the autoradiogram were detected by using preimmune immunoglobulin G (24; data not shown).

Similar results were found for all but the ldlA-650 cells when the anti-R antibody was used (Fig. 3, lanes 7 through 11). This antibody primarily recognizes disulfide bridge-dependent epitopes in the N terminus of the receptor (24). Anti-R did not precipitate the precursor form of the LDL receptor precursor as efficiently as did anti-C (Fig. 3, cf. lanes 1 and 7; data not shown). In addition to the major bands detected with anti-C, anti-R also detected a smaller protein (approximately 115 kDa) in the ldlA-7, RevA7, γRA7-64, and γRA7-89 cells but not in wild-type cells. This protein may be a rapidly formed degradation product of the abnormal LDL receptor in these cells.

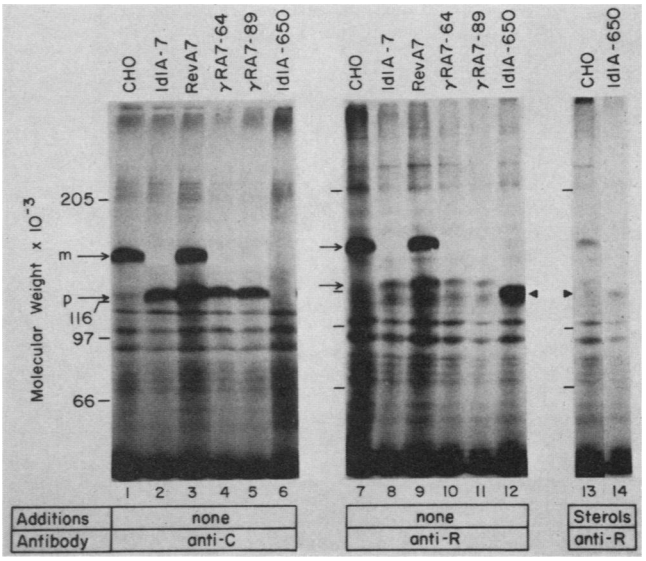

FIG. 3. LDL receptors synthesized by a family of related *ldlA* mutants. Each of the indicated cell types was incubated for 0.5 h in medium containing [^{35}S]methionine and then chased for 0.5 h in medium containing an excess of unlabeled methionine. Detergent-solubilized cell extracts were immunoprecipitated by using either an antibody (anti-C) which recognizes the C terminus of the LDL receptor (lanes 1 through 6) or an antibody (anti-R) which primarily recognizes the N-terminal region of the LDL receptor (lanes 7 through 14). To suppress LDL receptor synthesis, the cells used in lanes 13 and 14 were incubated for 21 h in medium containing cholesterol (12.5 μg/ml) and 25-hydroxycholesterol (0.5 μg/ml) before and during labeling. Immunoprecipitates were subjected to electrophoresis and autoradiography as described in Materials and Methods. Positions of the 125-kDa precursor (p) and the 155-kDa mature (m) forms of the LDL receptor (see the text) are indicated.

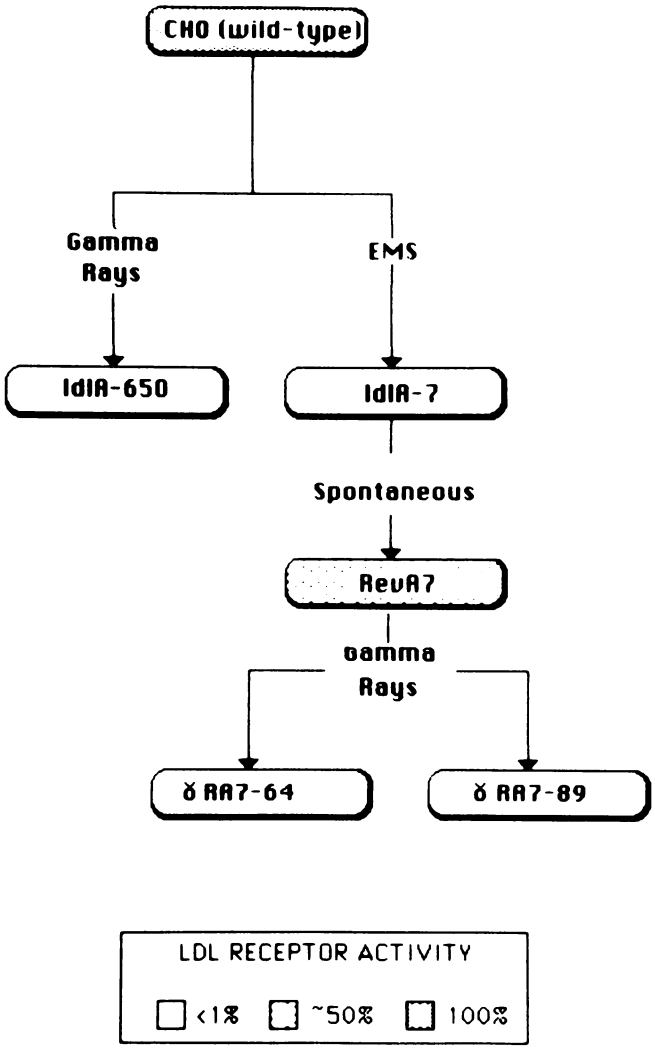

FIG. 2. Isolation of a family of related *ldlA* mutant and revertant cells. Cells were isolated by selecting for or against the expression of LDL receptor activity as discussed in the text. CHO K1 strain (wild-type), ldlA-7 (21), and RevA7 (21) cells have been described previously. The isolation of the gamma-ray mutants is described in Materials and Methods. The γRA7-64 and γRA7-89 cells were isolated from independently irradiated stocks of RevA7 cells. LDL receptor activity is expressed as a percentage [^{125}I]LDL degradation in wild-type cells (25).

Anti-R precipitated a 115- to 117-kDa protein (Fig. 3, arrowhead) from the ldlA-650 cells which was not seen in anti-C precipitates (cf. lane 12 with lane 6). Two observations demonstrated that this protein represented an abnormal form of the LDL receptor rather than an unrelated cross-reacting protein. First, the protein was not precipitated by preimmune immunoglobulin G from the same rabbit (see Fig. 4A, preimmune lanes). Second, as was the case for the synthesis of wild-type LDL receptors, synthesis of the 115- to 117-kDa protein in ldlA-650 cells was suppressed by preincubation of the cells with exogenous sterols (Fig. 3, cf. lanes 7 and 13 and lanes 12 and 14). Sterol-mediated transcriptional suppression is one of the hallmarks of LDL receptor synthesis (38). Taken together, these findings suggest that the abnormal receptor in ldlA-650 cells was apparently smaller than wild-type receptors, at least in part because of a truncation at the C terminus.

The defect in the ldlA-650 cells did not prevent the addition of high-mannose N-linked glycans to the receptor. When the cells were treated with tunicamycin, an inhibitor

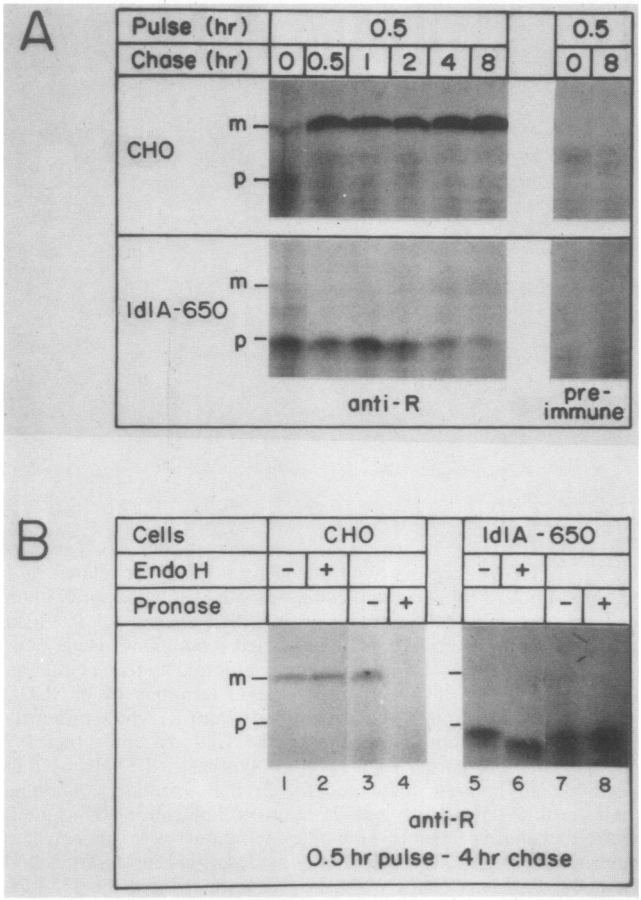

FIG. 4. LDL receptor processing in wild-type and ldlA-650 cells. (A) Wild-type and ldlA-650 cells were pulse-labeled for 0.5 h in medium containing [^{35}S]methionine and were either harvested immediately (0 chase) or chased for the indicated times in medium containing an excess of unlabeled methionine. Detergent-solubilized cell extracts were subjected to immunoprecipitation (anti-R or preimmune). (B) The indicated cell types were pulse-labeled for 0.5 h in medium containing [^{35}S]methionine and chased for 4 h in medium containing an excess of unlabeled methionine. Some of the cells were immediately harvested and subjected to immunoprecipitation (anti-R), followed by treatment with endoglycosidase H (lanes 2 and 6) or no treatment (lanes 1 and 5). The other monolayers were incubated with (lanes 4 and 8) or without (lanes 3 and 7) pronase (20 μg/ml, 20 min) and then harvested and subjected to immunoprecipitation (anti-R). In the experiment shown in panel B, wild-type cells were seeded at a lower density (31,000 cells per well) than were the ldlA-650 cells (150,000 cells per well). All immunoprecipitates (panels A and B) were prepared and analyzed by electrophoresis and autoradiography as described in Materials and Methods.

of N-linked glycosylation, the apparent size of the abnormal receptor decreased approximately 6 kDa (data not shown). To determine whether the posttranslational processing of this abnormal receptor was altered, we performed a series of pulse-chase experiments. While the receptors in wild-type cells underwent normally rapid conversion from 125-kDa endoglycosidase H-sensitive precursors to the 155-kDa endoglycosidase H-resistant mature receptors (24), the earliest detectable form of the receptor in ldlA-650 cells (115 to 117 kDa) was not further processed during an 8-h chase (Fig. 4A). Unlike the wild-type receptor, the ldlA-650 receptor remained endoglycosidase H sensitive for at least 4 h after synthesis (Fig. 4B, cf. lanes 1 and 2 to lanes 5 and 6). Thus, the high-mannose N-linked glycans on this mutant receptor were not processed to the complex form (23).

The mutant receptor was clearly less stable than the wild-type receptor (Fig. 4A). This instability could not be accounted for by secretion (no detectable 115- to 117-kDa receptor in the culture medium [data not shown]). When metabolically labeled cell extracts were separated into membrane and soluble fractions, virtually all of the wild-type receptor was found in the membrane fraction (data not shown). In contrast, most of the ldlA-650 receptor protein was found in the soluble fraction (data not shown). Unlike the wild-type receptor (Fig. 4B, lanes 3 and 4), the abnormal receptor was resistant to pronase for as long as 4 h after synthesis (lanes 7 and 8), indicating that it did not reach the cell surface (24).

LDL receptor gene structure in wild-type and mutant CHO cells. DNA was isolated from cells and analyzed by Southern blot hybridization. *Eco*RI digests of wild-type DNA contained a fragment of 4.8 kb which was detected by the Bov1518 probe (Fig. 5A, lane 1). The bands seen in digests of DNA from EMS-generated mutant ldlA-7 (Fig. 5A, lane 2) and revertant RevA7 (lane 3) appeared essentially identical in size and intensity to that in wild-type DNA. These results are consistent with the findings that EMS-generated mutations are often either point mutations or small deletions or insertions and rarely affect the hybridization patterns seen in Southern blots (36).

In contrast, the relative intensities of the analogous bands in the three mutants isolated after gamma irradiation (Fig.

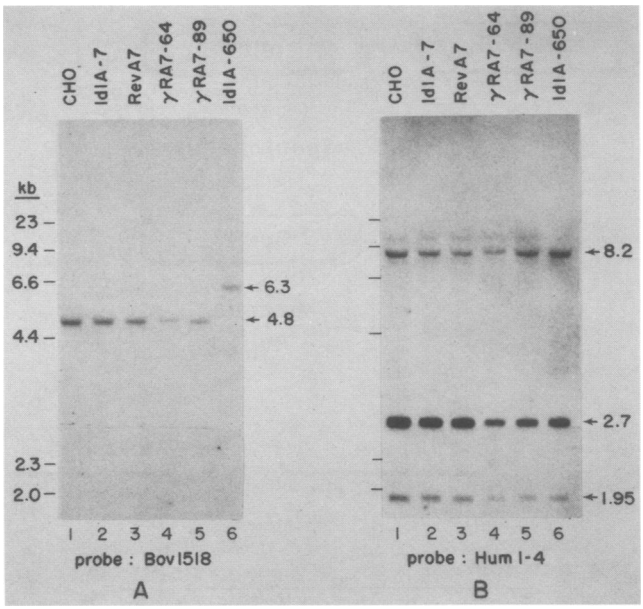

FIG. 5. Analysis of the LDL receptor genes in mutant and revertant *ldlA* cells. DNA (approximately 8 μg [see below]) from each of the indicated cell types was digested to completion with *Eco*RI, separated on a 1% agarose gel, and immobilized on a nylon filter. (A) The filter was hybridized with ^{32}P-labeled Bov1518, a bovine cDNA probe corresponding to the 3′ end of the coding region of the LDL receptor gene (Table 1). (B) The same filter was washed and hybridized to ^{32}P-labeled Hum1-4, a human cDNA probe corresponding to the 5′ end of the LDL receptor gene. The relative amounts of DNA loaded in each lane were determined by using an independent DNA probe (see Methods) and were found to be as follows: lane 1, 1.00; lane 2, 0.71; lane 3, 0.68; lane 4, 0.51; lane 5, 0.86; lane 6, 0.89.

TABLE 2. Hybridization intensity of LDL receptor gene fragments in related mutant and revertant CHO cells

Cell line	Relative intensity[a]					
	Bov1518	Hum1-4			Ham12	
	4.8 kb	1.95 kb	2.7 kb	8.2 kb	3.7 kb	8.2 kb
CHO (wild type)[b]	1.00 ± 0.19	1.00 ± 0.19	1.00 ± 0.16	1.00 ± 0.32	1.00 ± 0.20	1.00 ± 0.23
ldlA-7	1.03 ± 0.27	1.20 ± 0.34	1.13 ± 0.24	0.81 ± 0.23	ND	ND
RevA-7	0.92 ± 0.41	1.00 ± 0.27	1.16 ± 0.30	0.77 ± 0.29	ND	ND
γRA7-64	0.60 ± 0.23	0.54 ± 0.12	0.51 ± 0.08	0.70 ± 0.28	0.54 ± 0.09	0.91 ± 0.20
γRA7-89	0.64 ± 0.24	0.52 ± 0.18	0.46 ± 0.13	0.95 ± 0.34	0.36 ± 0.08	0.82 ± 0.15
ldlA-650[b]	0.50 ± 0.11[c]	0.68 ± 0.19	0.56 ± 0.12	1.03 ± 0.32	0.38 ± 0.11	0.95 ± 0.27

[a] The autoradiograms shown in Fig. 5A (probe Bov1518), 5B (probe Hum1-4), and 7 (probe Ham12) were scanned with a laser densitometer. The observed intensities for each band were measured and corrected for differences in DNA loading, as described in Materials and Methods. The relative intensity of each band is the ratio of the corrected intensity of that band to the corrected intensity of the corresponding band in wild-type CHO cells, plus or minus the standard deviation of the ratio (2). ND, Not determined.

[b] Values shown for bands detected by probes Bov1518 and Hum1-4 represent averages from two lanes on the same filter. Figure 5 shows only one of these two lanes.

[c] This band was 6.3 kb (Fig. 5A).

5A, lanes 4, 5, and 6) were approximately one-half that of wild type (Table 2, Bov1518 probe). The restriction fragment detected in ldlA-650 DNA by Bov1518 (6.3 kb) was significantly larger as well as less abundant (Fig. 5A, lane 6; Table 2) than that in wild-type DNA (4.8 kb). These results suggest that the LDL receptor gene in CHO cells is diploid and that each of these three mutants has a deletion affecting one of the two LDL receptor alleles (see Discussion). No evidence for restriction fragment-length polymorphisms in wild-type cells was obtained by using Southern blot analysis of DNA samples digested with restriction enzymes *Taq*I, *Xba*I, *Hin*dIII, *Eco*RI, *Bam*HI, or *Ban*II (Fig. 5 through 7 and data not shown). The correlation of the loss of LDL receptor expression with alterations affecting the LDL receptor gene in these three independently isolated *ldlA* mutants strongly suggests that the *ldlA* locus in CHO cells encodes the LDL receptor.

To determine the extent of the apparent deletions in these mutants, we used a probe (Hum1-4) corresponding to the 5′ end of the coding region of the gene (Table 1). This probe detected three major *Eco*RI restriction fragments in all of these cells (8.2, 2.7, and 1.95 kb; Fig. 5B). The intensities of the 2.7- and 1.95-kb bands were reduced by about 50% in the three gamma-ray-induced mutants (Fig. 5B, lanes 4, 5, and 6; Table 2) compared with those of wild-type cells. These reductions were similar to those observed in the bands detected by Bov1518 (Table 2) and were seen in other, independent Southern blots (data not shown).

Because the two γRA7 mutants produced abnormally processed but apparently full-length LDL receptors which were similar to those observed in the ldlA-7 and RevA7 cells from which they were derived (Fig. 3), one allele was probably not affected by gamma irradiation. Therefore, the reduction in hybridization intensity observed with probes which spanned the entire coding portion of the gene (Table 1; Fig. 5 through 7; data not shown) probably represents a large deletion in a single allele rather than smaller deletions in each of the two alleles.

When the 5′ probe was used, the ldlA-650 DNA appeared identical to DNA from the other two *ldlA* mutants isolated after gamma irradiation, suggesting that, in this region of the gene, one allele was deleted and the other allele was unaffected. We used probes Hum1314 and Hum1517 to locate more precisely the genetic defect in the remaining allele in the ldlA-650 cells. Hum1314 detected three bands (8.2, 4.8, and 3.7 kb) in *Eco*RI digests of wild-type DNA (Fig. 6, lane 1). The mobilities of the 8.2- and 3.7-kb bands were unchanged in the ldlA-650 DNA (Fig. 6, lane 2). The 4.8-kb fragment in wild-type DNA was detected by Hum1517 (lane 3) as well as Hum1314. This fragment appeared to be split into two new fragments in ldlA-650 DNA. One of the new fragments was 3.5 kb and was detected only by Hum1314 (Fig. 6, lane 2), while the other new fragment was 6.3 kb and was detected by Hum1517 (lane 4) and not by Hum1314 (lane 2). Thus, the single remaining *ldlA* allele in this mutant was disrupted between exons 13 and 17. This mutation may have been the result of a chromosomal rearrangement (e.g., a translocation or an inversion) and could account for the

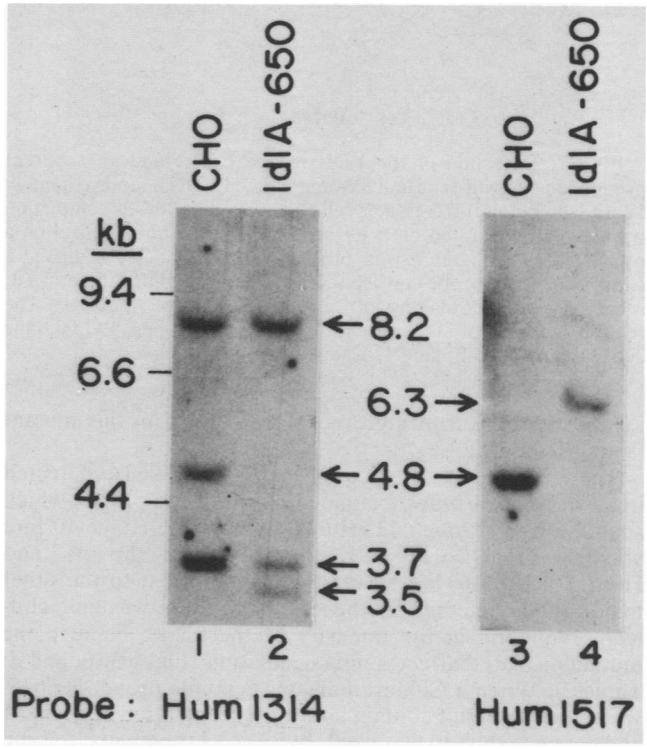

FIG. 6. Analysis of LDL receptor genes in wild-type and ldlA-650 cells. *Eco*RI digests of wild-type and ldlA-650 DNA were separated and transferred to the nylon filter described in the legend to Fig. 5. The filter was washed and hybridized with ^{32}P-labeled Hum1314 (lanes 1 and 2) and then washed and hybridized with ^{32}P-labeled Hum1517 (lanes 3 and 4). The relative amounts of DNA loaded (see Methods) were 1.00 (lane 1) and 0.81 (lane 2).

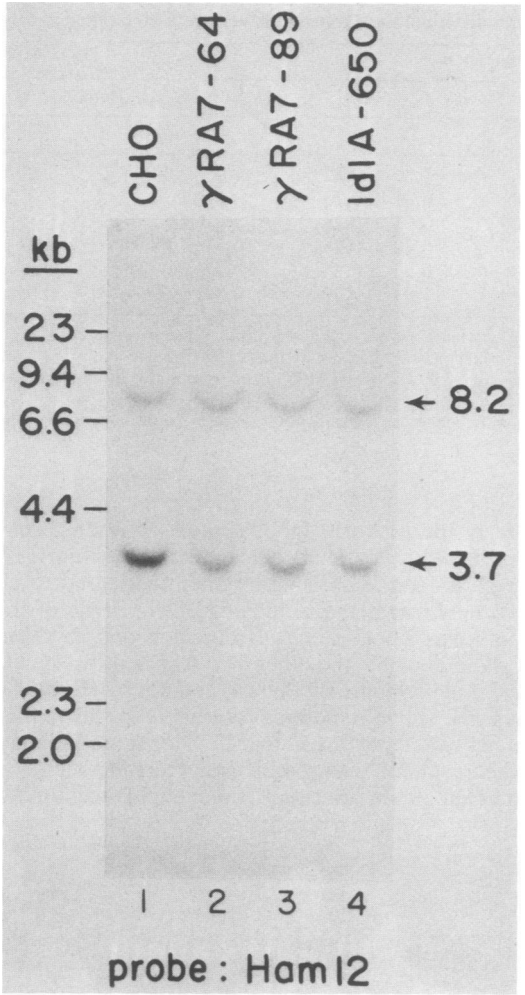

FIG. 7. Detection of the LDL receptor gene and an apparent pseudogene in wild-type and *ldlA* mutant cells. DNA (approximately 12 μg [see below]) from each cell type was digested to completion with *Eco*RI, separated on a 1% agarose gel, and immobilized on a nylon filter. This filter was probed with ^{32}P-labeled Ham12, a Chinese hamster probe corresponding to exon 12 of the human LDL receptor gene and flanking intron sequences (Fig. 1; Table 1). The relative amounts of DNA loaded were as follows: lane 1, 1.00; lane 2, 1.32; lane 3, 1.42; lane 4, 1.38.

synthesis of the truncated receptor detected in this mutant (see Discussion).

The 8.2-kb band detected by Hum1314 was also detected in all of the cell types examined by all of the probes which span exons 1 through 12 (Hum1-4 [Fig. 5B], Hum8-10 [not shown], Ham12 [Fig. 7], Bov1214 [not shown], and Hum1314 [Fig. 6, lanes 1 and 2]). In contrast to the other major bands detected by these probes, there was no significant reduction in the intensity of the 8.2-kb band in the mutants isolated after gamma irradiation (Fig. 5B, 6, and 7; Table 2). When a Chinese hamster genomic probe (Ham12) which did not itself contain an *Eco*RI recognition site (Fig. 1 and data not shown) was used, two *Eco*RI fragments (8.2 and 3.7 kb [Fig. 7]) were detected. Only the 3.7-kb band exhibited reduced intensity in the mutants isolated after gamma irradiation (Fig. 7; Table 2). These observations raised the possibility that the 8.2-kb band did not represent a portion of the LDL receptor gene. A series of experiments ruled out the possibility that the appearance of the 8.2-kb band was an artifact of the cloning procedures used, the enzyme digests performed, or the specific cell line from which DNA was derived (data not shown).

The 8.2-kb band was highly homologous to the LDL receptor gene. For example, it was detected under hybridization and wash conditions too stringent to allow the detection of the human LDL receptor gene by Ham12 (65°C, 0.1× SSPE [data not shown]). Thus, it probably did not represent an unrelated gene in the epidermal growth factor precursor superfamily of genes, the members of which are less homologous to each other than are LDL receptor genes isolated from different species (Fig. 1) (37, 48, 53). The 8.2-kb band was not recognized by probes spanning exons 15 to 18 (Bov1518 [Fig. 5B] and Hum1517 [Fig. 6, lanes 3 and 4]). All of the bands that were detected by these 3′ probes had reduced intensities in DNA from the mutants isolated after gamma irradiation (Table 2 and data not shown). These findings strongly suggest that there may be an incomplete, possibly processed, LDL receptor pseudogene in cultured Chinese hamster cells.

DISCUSSION

In this study, we have constructed a family of mutants (Fig. 2) to determine directly the identity of the *ldlA* locus. This locus is one of four required for the expression of LDL receptor activity in CHO cells (21, 27). Since gamma irradiation of CHO cells frequently induces readily detectable genetic changes (14), our approach was to isolate gamma-ray-induced *ldlA* mutants and determine whether there was a correlation between changes in the expression of LDL receptors and changes in the structure of the LDL receptor gene. Three independent receptor-negative (<1% of wild-type LDL receptor activity) *ldlA* mutants, γRA7-64, γRA7-89, and ldlA-650, which were isolated after gamma irradiation, were examined in detail.

We used Southern blot analysis to detect alterations in the structures of the LDL receptor genes in these mutants. Densitometric scanning of these blots revealed that all three mutants isolated after gamma irradiation contained approximately half of the number of copies of the LDL receptor gene found in wild-type cells. While these data suggest that the *ldlA* locus is diploid, the uncertainties in the measurements (Table 2) raise the possibility that there could be more than two *ldlA* alleles. However, this appears to be unlikely for several reasons. (i) The frequencies at which new *ldlA* mutants were isolated after EMS treatment of wild-type cells and a heterozygous revertant (RevA7 [see below]) were 1.3×10^{-6} and 3.2×10^{-4}, respectively (21). These frequencies were essentially identical to the frequencies at which adenine phosphoribosyltransferase-negative (APRT$^-$) mutants were isolated after EMS treatment of cells which contained either two (diploid wild-type) or one (hemizygous mutant) copies of the *aprt* gene (36). (ii) Heterozygous RevA7 cells expressed approximately 50% of wild-type receptor activity. Analogous activities (approximately 50% of wild type) have been reported for heterozygous *aprt* (36) and *dhfr* (49) mutants. (iii) All of our structural and functional data (see above and references 24 and 40) are consistent with the presence of two *ldlA* alleles in wild-type cells. (iv) We are not aware of any examples of genes in wild-type CHO cells for which there are more than two alleles. Therefore, we conclude that, although the CHO cell line is hypodiploid (41, 42, 46), the *ldlA* locus is diploid, and the three *ldlA* mutants isolated after gamma irradiation had deletions affecting one of the two alleles.

In conjunction with our previous work (21, 24, 40), these results also establish that the diploid *ldlA* locus is the structural gene for the LDL receptor in CHO cells. Since *ldlA* mutants have defects in the LDL receptor gene, they are analogous to cell lines derived from patients with the homozygous form of familial hypercholesterolemia (12). Many of the CHO *ldlA* mutants (such as ldlA-650 [see below]) produce LDL receptors which appear similar to receptors previously described in human familial hypercholesterolemic mutants, while others (such as ldlA-7 [see below]) are clearly different. In addition, Chinese hamster cells appear to contain a truncated LDL receptor pseudogene which was not altered in any of the *ldlA* mutants examined and did not contain sequences homologous to the 3′ end of the LDL receptor gene.

The derivation of mutants γRA7-64 and γRA7-89 is shown in Fig. 2. These mutants were isolated after gamma irradiation of RevA7, a revertant with 50% of wild-type LDL receptor activity. This revertant arose spontaneously from ldlA-7, a receptor-negative mutant derived from EMS-treated wild-type cells. The ldlA-7 cells synthesized an abnormally processed, unstable LDL receptor protein (Fig. 3) (24). The distinctive, continuous processing of these receptors distinguishes this class of mutant receptor from those in previously described human familial hypercholesterolemic cells (12, 24, 27). RevA7 cells synthesized two distinct forms of LDL receptor: (i) abnormal receptors indistinguishable from those produced by ldlA-7 (Fig. 3) (24) and (ii) functional receptors indistinguishable from those produced by wild-type cells. Both γRA7 cell strains synthesized only the abnormal form. Thus, the selection of *ldlA* mutants from gamma-irradiated RevA7 cells resulted in the isolation of cells in which only one allele, the functional one, appeared to have been affected by gamma-ray mutagenesis. These findings explain the substantially higher frequencies of EMS-dependent *ldlA* mutant isolation from RevA7 cells relative to that obtained from wild-type cells (21). Only the single functional *ldlA* allele in the RevA7 cells need be mutated to produce a receptor-negative clone.

The ldlA-650 mutant cells were derived directly from gamma-irradiated wild-type cells. Because the frequency at which *ldlA* mutants were isolated after gamma-ray treatment of wild-type cells was very low (see Methods), we cannot be certain that the abnormalities of the *ldlA* alleles in the ldlA-650 cells were the direct consequences of gamma irradiation. The precise nature of the mutations in the LDL receptor gene in the ldlA-650 cells remains uncertain. The data are consistent with the deletion of one allele (not necessarily the same allele which was deleted in the γRA7 cells) and a chromosomal rearrangement affecting the second allele. Chromosomal rearrangements have frequently been observed after gamma irradiation (see reference 52 for a review). Other possible defects (e.g., insertions) have not been ruled out.

The disruption in the single remaining allele in these cells occurred within the region corresponding to human exons 13 to 17 (see above and reference 12). The human LDL receptor gene exon 15 encodes a domain of the polypeptide chain which is heavily modified by the addition of clustered O-linked glycans (4). The posttranslational modification of these O-linked carbohydrates accounts for a large part of the shift in apparent molecular weight observed in the transition from the precursor to the mature form of the receptor (4, 24). Exons 16 and 17 encode the transmembrane domain and flanking regions. Exon 18 encodes the remaining portion of the cytoplasmic domain of the receptor and also contains a large 3′ untranslated region. The ldlA-650 cells produced a truncated receptor protein which was lacking the normal C terminus and was not membrane-associated. In addition, the oligosaccharide chains linked to the precursor form of this mutant receptor (including high-mannose N-linked chains) were not further processed. Thus, while newly synthesized ldlA-650 LDL receptors, like wild-type receptors, appear to undergo rough endoplasmic-reticulum-associated glycosylation, they may not be properly transported to the medial and *trans* Golgi compartments for normal posttranslational processing.

Mutant LDL receptors lacking the C-terminal domain have previously been observed in cells (FH 274) isolated from patients with familial hypercholesterolemia (12, 31). In these cells, the coding portions of exons 16 through 18 have been deleted (31). Fibroblasts bearing this mutation synthesize truncated LDL receptors which undergo apparently normal posttranslational modification (12). Most of these receptors are secreted (31); however, some remain tightly associated with the plasma membrane and can mediate LDL binding but not endocytosis (13). A different human LDL receptor mutation, R-100, results in the synthesis of a truncated receptor similar to that synthesized by the ldlA-650 cells in that it is not processed and cannot be detected by using an antibody which recognizes the C terminus (12; D. Russell, M. Brown, and J. Goldstein, personal communication). The genetic basis of the defect in the R-100 cells has not yet been determined. Detailed comparisons of these three mutants (ldlA-650, FH 274, and R-100) may help to identify those structural features of the receptor required for normal intracellular transport. Since *ldlA* mutants efficiently express and process exogenous LDL receptor genes (40), the introduction into these mutants of cloned receptor genes with in vitro-generated mutations may help define those structural features of the receptor required for normal processing, transport, and function (6, 12).

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