rapid communication

Tissue expression studies on the mouse acyl-CoA: cholesterol acyltransferase gene (*Acact*): findings supporting the existence of multiple cholesterol esterification enzymes in mice

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Abstract Cholesterol esterification is involved in the regulation of cellular cholesterol content and has been hypothesized to play a role in important physiologic processes including intestinal cholesterol absorption, hepatic lipoprotein production, and macrophage foam cell formation in atherosclerotic lesions. Although intitial studies of the mouse acyl CoA:cholesterol acyltransferase gene (*Acact*) suggested that its gene product was responsible for cholesterol esterification in most tissues, we observed recently that *Acact*$^{-/-}$ mice have only tissue-specific reductions in cholesterol esterification. To better understand the role of *Acact* in cholesterol esterification, we used in situ hybridization and immunoblotting to perform tissue expression studies in wild-type mice. We found high levels of *Acact* expression in steroidogenic tissues, sebaceous glands, and atherosclerotic lesions, but not in the liver or the small intestine. These data support the hypothesis that multiple cholesterol esterification enzymes exist in mammals and that another enzyme is likely to be responsible for cholesterol esterification activity in mouse liver and intestine.—**Meiner, V., C. Tam, M.D. Gunn, L-M. Dong, K. H. Weisgraber, S. Novak, H. M. Myers, S. K. Erickson, and R. V. Farese, Jr.** Tissue expression studies of the mouse acyl-CoA:cholesterol acyltransferase gene (*Acact*): findings supporting the existence of multiple cholesterol esterification enzymes in mice. *J. Lipid Res.* 1997. **38:** 1928–1933.

Supplementary key words cholesteryl esters • macrophage • adrenal gland • preputial gland • atherosclerosis • skin

Cholesterol and fatty acids in cells are converted to cholesteryl esters in a reaction catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) (1–3). Cholesterol esterification activity has been demonstrated in many different mammalian tissues, including liver, small intestine, adrenal glands, brain, and macrophage-rich atherosclerotic lesions (1–3). Using sensitive techniques such as ribonuclease protection assays (4, 5), the initial studies of the cloned ACAT gene (5, 6) demonstrated wide-spread tissue expression of ACAT mRNA, suggesting that this gene product was responsible for cholesterol esterification in most tissues. However, we recently disrupted the mouse ACAT gene (*Acact*) (7) and observed tissue-specific reductions in cholesterol esterification: cholesteryl ester levels were markedly reduced in the adrenal glands and macrophages but were normal in the liver. Also, intestinal cholesterol absorption was not reduced in *Acact*$^{-/-}$ mice. These studies suggested that mammals, like yeast (8), may have multiple sterol esterification enzymes. In support of this, cholesterol esterification activity in different tissues has been shown previously to respond differently to chemical modification (9) or an ACAT inhibitor (10).

To understand better the role of *Acact* in cholesterol esterification, we used in situ hybridization and immunoblotting, the latter using a new antiserum that recognizes the mouse *Acact* protein, to perform expression studies in wild-type mice. We also examined for the presence of *Acact* mRNA in atherosclerotic lesions of apoli-

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; apo, apolipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST, glutathione-S-transferase.

poprotein (apo) E-deficient mice. The current findings support the hypothesis that the mouse *Acact* gene product plays a major role in cholesterol esterification in steroidogenic tissues, sebaceous glands, and atherosclerotic lesions but not in the liver or the small intestine.

MATERIALS AND METHODS

Mice

Wild-type male mice of mixed DBA/2 and C57BL/6 genetic background were used for in situ hybridization studies. Sections of atherosclerotic lesions were obtained from apoE-deficient mice (11) (C57BL/6 genetic background) fed a chow diet for several months. Mice were perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline. After overnight fixation, the tissues were embedded in paraffin and sectioned at 5-μm intervals under RNase-free conditions. Mice used for immunoblotting studies were either wild-type or $Acact^{-/-}$ mice (2) (mixed C57BL/6 and 129/Sv background). Peritoneal macrophages were harvested 3 days after intraperitoneal injection of 3 ml of 3% thioglycollate as described (12). All mice were housed in a pathogen-free, transgenic barrier facility in accordance with UCSF Animal Care Facility guidelines.

In situ hybridization

The *Acact* probe was synthesized from a 501-bp cDNA fragment (nucleotides 804–1304 of the mouse *Acact* cDNA sequences (5)) spanning the 5′ coding region. Plasmids were linearized with appropriate restriction enzymes and transcribed with T7 or T3 RNA polymerase (Boehringer-Mannheim) and ^{35}S-labeled UTP (New England Nuclear) to generate antisense or sense radiolabeled-RNA probes. In situ hybridization was performed using a modification of published procedures (13). Tissue sections were deparaffinized, fixed in 4% paraformaldehyde in phosphate-buffered saline, and treated with proteinase K. After washing in 0.5 × SSC, the sections were covered with hybridization solution (50% deionized formamide, 0.3 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1 × Denhardt's solution, 10% dextran sulfate, and 20 mM dithiothreitol) and hybridized for 1–3 h at 55°C. ^{35}S-labeled antisense or sense RNA probes (300,000 cpm/slide) were added to the hybridization solution, and the incubation continued for 12–18 h at 55°C. After hybridization, the sections were washed for 20 min in 2 × SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA, treated with RNase A (20 μg/ml) for 30 min at room temperature, and washed at high stringency (0.1 × SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA) for 2 h at 60°C. The sections were dehydrated, dipped in photographic emulsion NTB_2 (Kodak), and stored at 4°C. After 8 weeks of exposure, the sections were developed and counterstained with hematoxylin and eosin.

Antibody generation and immunoblotting

The glutathione-S-transferase (GST) fusion protein expression vector pGEX3X was used to express the amino-terminal 120 residues of the mouse *Acact* protein in *Escherichia coli* as described (14). The GST–ACAT protein was purified by glutathione agarose affinity chromatography (14), and the intact fusion protein was used to prepare antiserum in pathogen-free rabbits. $Acact^{+/+}$ or $Acact^{-/-}$ male mice (n = 3 per group) were anesthetized with isofluorane, and tissues were dissected into normal saline on ice. Whole tissues were homogenized in 0.25 M sucrose and 1 mM EDTA, pH 7.4, in a Potter-Elvehjem homogenizer (10 strokes at moderate speed). Aliquots (50 μg) were analyzed by immunoblot as described (15). Alternatively, mouse tissues were homogenized in 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100. In this case, an equal volume of homogenization buffer containing 10% SDS was then added to protein aliquots (final concentration = 5% SDS); the samples were heated to 65°C for 20 min, and protein aliquots (75 μg) were subjected to SDS-PAGE with 10% gels. For all immunoblots, the separated proteins were transferred to nylon membranes and immunoblotted with the antirabbit antiserum specific for mouse *Acact*. Binding of the antibody was detected with enhanced chemiluminescence techniques (Amersham).

RESULTS

The tissue expression pattern of *Acact* mRNA was studied in adult male mice by in situ hybridization with a 501-bp antisense probe (derived from the 5′ end of the *Acact* coding sequence). High signal levels for the antisense probe corresponding to *Acact* mRNAs were observed in the preputial glands, sebaceous glands of skin, and adrenal cortex (**Fig. 1**). In each case, a sense probe used as a negative control showed no specific signal, demonstrating that the antisense probe was specific for *Acact* mRNA. The positive signal in the adrenal cortex was localized to the zona fasciculata. In contrast, specific signals for *Acact* mRNA were not detected in the liver or small intestine (Fig. 1) or in other tissues sam-

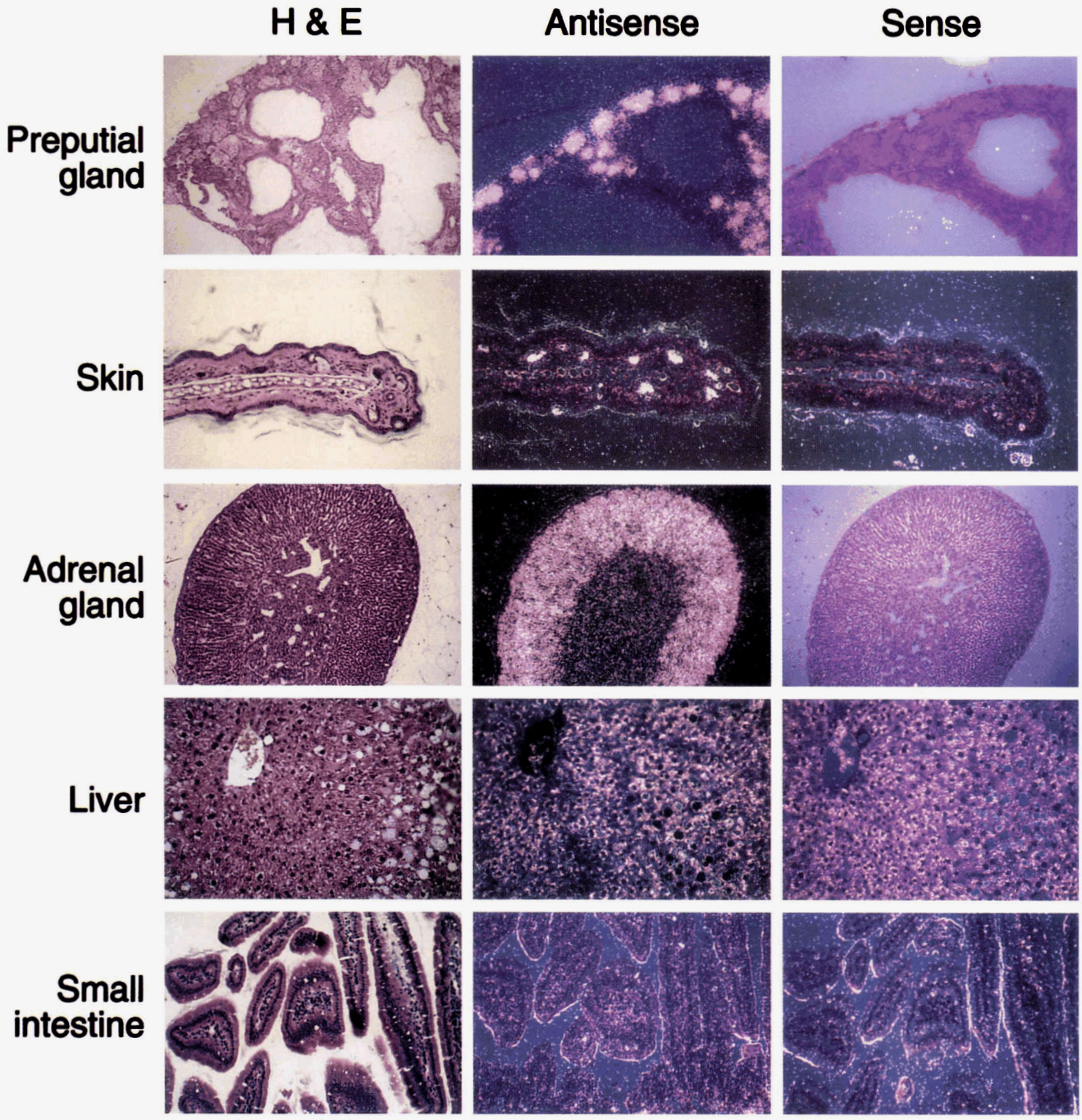

Fig. 1. *Acact* mRNA expression in normal mouse tissues determined by in situ hybridization. Sections from preputial gland, skin, adrenal gland, liver, and small intestine were prepared in RNase-free conditions and probed with either antisense or sense probes from the *Acact* cDNA as described in Materials and Methods. Photomicrographs were taken at 200× magnification for all sections, except liver (400×).

pled (brain, testis, pancreas, spleen, heart, lung, kidney, and eye) (not shown).

In situ hybridization also demonstrated *Acact* mRNA in atherosclerotic lesions of apoE-deficient mice (**Fig. 2**). The intense signal was localized to the region of intimal thickening, consistent with a macrophage foam cell distribution.

The tissue distribution of the *Acact* protein was determined by immunoblotting with a rabbit antiserum generated from immunization with the amino-terminal 120

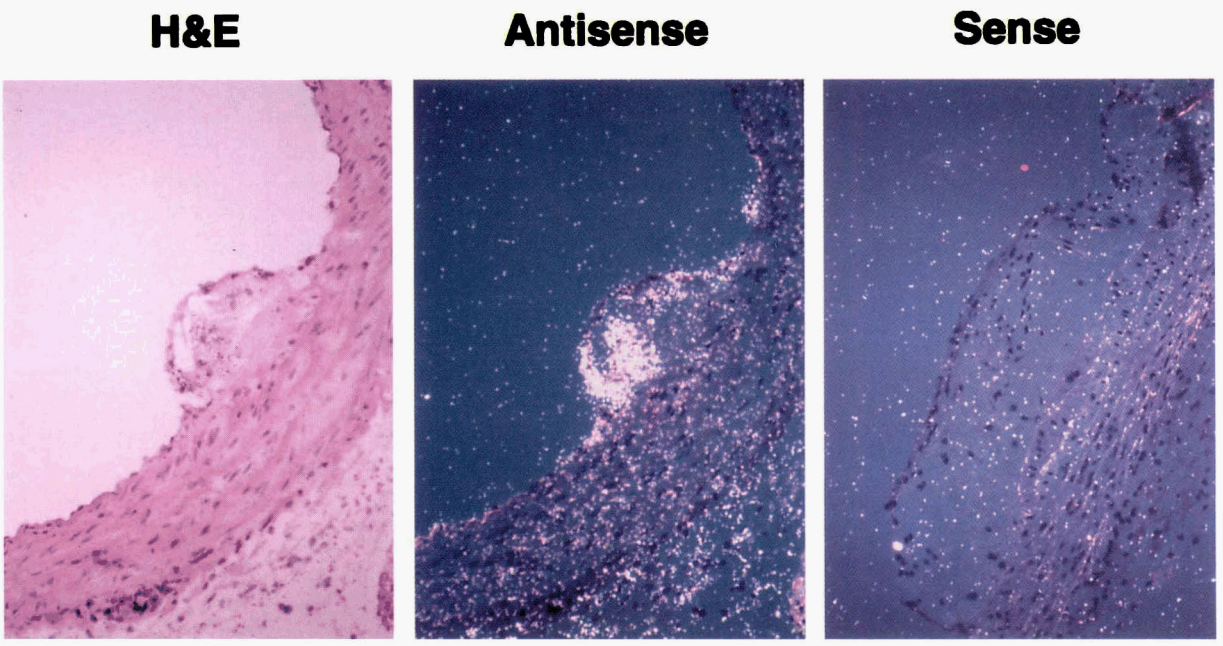

Fig. 2. Presence of *Acact* mRNA in atherosclerotic lesions determined by in situ hybridization. Sections of atherosclerotic lesions from apoE-deficient mice were probed with antisense or sense cDNA probes as described in Materials and Methods. Magnification, 200×.

amino acids of the mouse *Acact* protein. A 46-kDa protein was abundantly expressed in homogenates from the adrenal glands and the testis of wild-type mice (**Fig. 3**) but was absent in homogenates from *Acact*$^{-/-}$ mice, indicating that this band corresponds to the *Acact* gene product. This protein was present in smaller amounts in kidney and brain homogenates, and it was barely (and variably) detectable in the liver and absent in the small intestine. Abundant expression was also observed in homogenates of the preputial gland and peritoneal macrophages (**Fig. 4**). In other experiments (not shown), the fractionation of liver homogenates into subcellular components demonstrated the *Acact* protein primarily in microsomes and nuclear membranes but not in mitochondrial or cytosolic fractions.

DISCUSSION

The initial cloning and analysis of the mouse ACAT gene, *Acact*, demonstrated wide-spread tissue expression of *Acact* mRNA in all tissues (4, 5), suggesting that the *Acact* gene product was responsible for cholesterol esterification in most tissues. The current results indicate, however, that *Acact* may play a predominant role

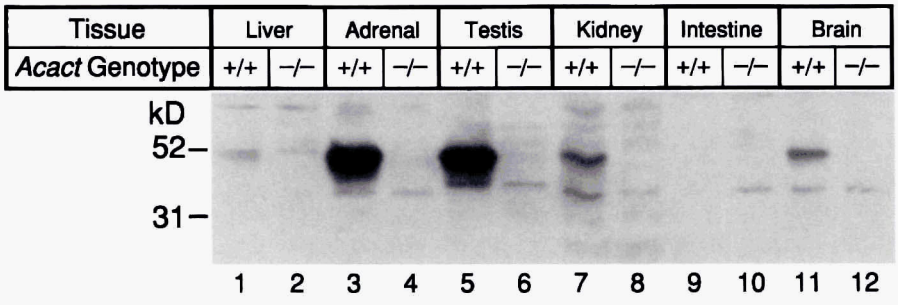

Fig. 3. Distribution of *Acact* protein in mouse tissues determined by immunoblot. Aliquots (50 μg) of tissue homogenates from wild-type or *Acact*$^{-/-}$ mice (7) were subjected to SDS-PAGE. Separated proteins were transferred to a nylon membrane and blotted with antiserum generated against the amino-terminus of the mouse *Acact* protein as described in Materials and Methods. The mouse *Acact* protein migrates with an apparent molecular mass of ~46 kDa.

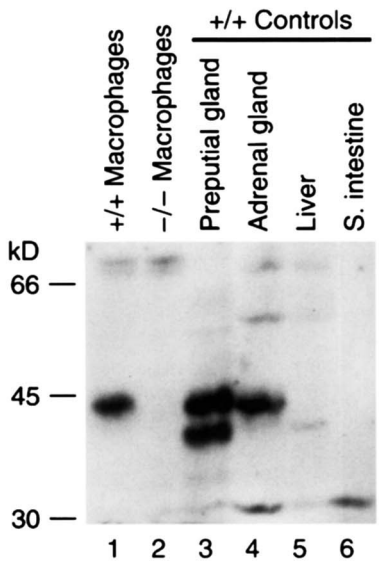

Fig. 4. *Acact* protein expression in peritoneal macrophages determined by immunoblot. Peritoneal macrophages were harvested from wild-type or *Acact*$^{-/-}$ mice as described (11), and protein homogenates (75 μg) were analyzed by immunoblotting as described in Materials and Methods. The intense band of lower molecular mass (~40 kDa in the preputial gland sample (lane 3) is not observed routinely and probably represents a degradation product in this sample.

only in selected tissues; *Acact* mRNA and protein were expressed at high levels in steroidogenic tissues, sebaceous glandular tissues, and atherosclerotic lesions but at low or undetectable levels in the liver and the small intestine. These results are in agreement with tissue-specific reductions in cholesterol esterification, primarily in the adrenal cortex and macrophages but not in the liver, observed in *Acact*$^{-/-}$ mice (7).

The presence of *Acact* mRNA and protein in the adrenal glands is consistent with the known function of cholesterol esterification in providing storage pools of cholesteryl esters in cytosolic droplets for steroidogenesis. *Acact* protein was also detected in testes homogenates. It is likely that the testicular expression corresponds to Leydig cells, which contain cytosolic cholesteryl ester droplets (16) and synthesize testosterone. The absence of positive signal in the testes in our in situ hybridization experiments probably resulted from a lack of sensitivity of this technique for detecting low levels of *Acact* mRNA. Although not studied here, the ovary expresses high levels of *Acact* mRNA (5), and we have detected the protein in this tissue by immunoblotting (V. Meiner and R. Farese, unpublished observations).

The high levels of *Acact* mRNA and protein expression in the preputial gland in this study confirm previous observations for this tissue (5). A new finding from this study is that *Acact* mRNA also is highly expressed in sebaceous glands of the skin. Both preputial and sebaceous glands are characterized by secretions that are rich in waxes (long-chain alcohol:fatty acid esters) and cholesteryl esters, although the function of cholesteryl esters in these secretions is unknown. The preputial gland secretions are thought to play a role in the male reproductive behavior of mice (17). Cholesterol esterification activity has been demonstrated previously in preputial glands (18) and mammalian skin (19). The finding of *Acact* mRNA in both of these glandular tissues suggests a common role for cholesteryl esters in sebaceous secretions.

High levels of *Acact* mRNA were found in atherosclerotic lesions, in agreement with high levels of cholesterol esterification activity observed in such lesions (3). It is likely that the distribution of *Acact* mRNA reflects that of macrophage foam cells; however, colocalization studies with *Acact*-specific and macrophage-specific antibodies will be needed to confirm this hypothesis. In support of this conclusion, we observed *Acact* protein expression in macrophages harvested from the peritoneum. The demonstration of *Acact* mRNA in atherosclerotic lesions, together with recent observations that the inactivation of *Acact* leads to diminished cholesteryl ester accumulation in macrophages (7), suggests that *Acact* deficiency and a resultant decrease in macrophage foam cell formation could affect the development of atherosclerotic lesions. Studies to address this question are in progress.

We found little evidence supporting a significant role for the *Acact* gene product in cholesterol esterification in mouse liver or intestine. These tissues secrete cholesteryl ester-containing lipoproteins, and cholesterol esterification has been hypothesized to play a role in physiologic processes related to these tissues, such as intestinal cholesterol absorption and hepatic synthesis and secretion of very low density lipoproteins. Both tissues have cholesterol esterification activity that has been well characterized (1–3). The lack of evidence for *Acact* expression in these tissues and the normal hepatic cholesterol esterification and normal intestinal cholesterol absorption in *Acact*$^{-/-}$ mice (7) suggest that the *Acact* gene product does not play a major role in cholesterol esterification in these tissues in mice. The current data support the hypothesis that multiple cholesterol esterification enzymes exist in mammals, one of which may be responsible for the activities in the mouse liver or intestine. We have recently identified two *Acact* homologues in the expressed sequence tag human cDNA databases (accession numbers R07932 and R10272), which may be additional cholesterol esterification enzymes.

We thank Yang He, Ricky Quan, and Steven Lear for expert technical assistance; Robert Mahley and Stephen Young for helpful comments on the manuscript; John Carroll and Amy Corder for graphics; Gary Howard and Stephen Ordway for editorial assistance; and Angela Chen for manuscript preparation. This work was supported by the J. David Gladstone Institute and an American Heart Association Grant-in-Aid (#95-239) (R.V.F.); Daiichi Pharmaceutical Co., Ltd. (C.T. and M.D.G.); and NIH grant HL52069 and the Department of Veterans Affairs (S.K.E.).

Manuscript received 1 July 1997.

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