Levels of Messenger Ribonucleic Acid Encoding Cholesterol Side-Chain Cleavage Cytochrome P-450, 17α-Hydroxylase Cytochrome P-450, Adrenodoxin, and Low Density Lipoprotein Receptor in Bovine Follicles and Corpora Lutea throughout the Ovarian Cycle*

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To investigate the molecular basis for the pattern of ovarian steroid production during the bovine estrous cycle, the relative levels of mRNA specific for cholesterol side-chain cleavage cytochrome P-450, 17α-hydroxylase cytochrome P-450, adrenodoxin, and low density lipoprotein receptor were determined in ovarian antral follicles of differing size (<3–18 mm) and corpora lutea from the early, early-mid, late-mid, and regressionary stages. Total and poly(A)$^+$ RNA was size-fractionated on agarose-formaldehyde gels, transferred to nylon filters and hybridized to specific ^{32}P-labeled probes. The levels of mRNAs for the rate-limiting enzymes in the conversion of cholesterol into progesterone, namely cholesterol side-chain cleavage cytochrome P-450 and its electron donor, adrenodoxin, were higher in corpora lutea than in follicles. Conversely the levels of mRNA specific for the key regulatory enzyme in the conversion of pregnenolone or progesterone to androgen, namely 17α-hydroxylase cytochrome P-450, were high in all antral follicles examined but were low in young corpora lutea and undetectable in more mature corpora lutea. Low density lipoprotein receptor mRNA was detectable in antral follicles and corpora lutea but the levels were greater in corpora lutea. These results suggest that the pattern of changes in steroid hormone biosynthesis during the bovine estrous cycle and in the ovarian content of steroidogenic enzymes is related to and probably dependent upon the pattern of change in levels of mRNAs for steroidogenic enzymes and related proteins. (Molecular Endocrinology 1: 274–279, 1987)

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INTRODUCTION

The bovine ovary secretes a number of different steroid hormones, principally estradiol and progesterone (1). The relative and absolute amounts secreted change throughout the estrous cycle in a precisely coordinated and episodic fashion in concert with growth and development of follicles and corpora lutea (2). The rates of secretion of steroid hormones are related to the follicular and luteal content of cholesterol side-chain cleavage cytochrome P-450 (P-450$_{scc}$), adrenodoxin, and 17α-hydroxylase cytochrome P-450 (P-450$_{17\alpha}$) (3) which are key regulatory enzymes in the conversion of cholesterol to progesterone, and the conversion of pregnenolone or progesterone into androgen, respectively. Cytochrome P-450$_{scc}$ is present in the theca interna of follicles and to a variable degree in the membrana granulosa (4). After ovulation there is a dramatic increase in the specific contents of both P-450$_{scc}$ and adrenodoxin in the corpus luteum (3), and P-450$_{scc}$ is present in small and large luteal cells (5). This increase presumably accounts for the large increase in the amount of progesterone secreted as a follicle ovulates and develops into a corpus luteum. In addition, the content of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis, is substantially greater in corpora lutea (6) thus providing more cholesterol, via *de novo* synthesis, as substrate for progesterone biosynthesis. In contrast, the content of P-450$_{17\alpha}$, present in the theca interna (4), declines to undetectable levels after ovulation (3), thus accounting for a reduction in the secretion of androgens, and also estrogen, after ovulation.

In order to understand more fully the mechanisms by which the contents of steroidogenic enzymes change

throughout the estrous cycle, we have measured the relative levels of mRNAs encoding cytochrome P-450_{scc}, P-$450_{17\alpha}$, adrenodoxin (the electron donor for cytochrome P-450_{scc}), and the low density lipoprotein (LDL) receptor. The LDL receptor, like HMGCoA reductase, is a key factor in the provision of substrate cholesterol for progesterone biosynthesis (7).

RESULTS

The levels of mRNA for P-450_{scc}, adrenodoxin, P-$450_{17\alpha}$ and LDL receptor in equal amounts of RNA from ovarian stroma, follicles, and corpora lutea were determined by Northern blotting analysis and the results are shown in Figs. 1–4, respectively. The levels of each specific mRNA species present at a particular stage of follicular or luteal development varied from one individual sample to another. Whether this reflected individual variation or difficulty in determining the exact stage of the tissues is not clear, but nevertheless the qualitative trends were readily apparent.

Cytochrome P-450_{scc} and Adrenodoxin

It was found (Fig. 1) that a plasmid containing part of the coding region of bovine P-450_{scc} (pBSCC-2) hybridized to total RNA about the size of 18S ribosomal RNA from corpora lutea of stages I, II, and III. When poly(A)$^+$ RNA was prepared from stage II corpora lutea, pBSCC-2 hybridization was observed, but not to poly(A)$^-$ RNA (data not shown). Hybridization was not detected with RNA from any of the large follicles tested nor from corpora lutea of stage IV, the regressionary phase. A plasmid containing the coding region of bovine adrenodoxin (pAdx4) (*Bam*HI-*Bam*HI) hybridized to RNA smaller than 18S ribosomal RNA, from corpora lutea of classes II and III (Fig. 2). Weaker hybridization was observed on autoradiograms exposed for longer periods of time, to RNA from corpora lutea of stage I but not that from follicles of various sizes nor ovarian cortex.

Cytochrome P-$450_{17\alpha}$

It was observed that a plasmid containing the coding region of bovine P-$450_{17\alpha}$ (pcD17α-2) (*Pst*I-*Pst*I) hybridized to RNA about the size of 18S ribosomal RNA from all sizes of follicles (<3–11 mm) examined and that the degree of hybridization was similar between the follicles of different sizes (Fig. 3). No hybridization was observed with RNA from the ovarian cortex or corpora lutea of stages II or III. However, weak hybridization was observed with one of the two young stage I corpora lutea.

LDL Receptor

Hybridization to RNA larger than 28S ribosomal RNA was detected in follicles and corpora lutea using DNA complementary to mRNA encoding the LDL receptor (Fig. 4). No hybridization was detected with RNA from ovarian cortex and the degree of hybridization was greater in corpora lutea of stages I, II, and III than in any of the sizes of follicles (<3–11 mm) examined.

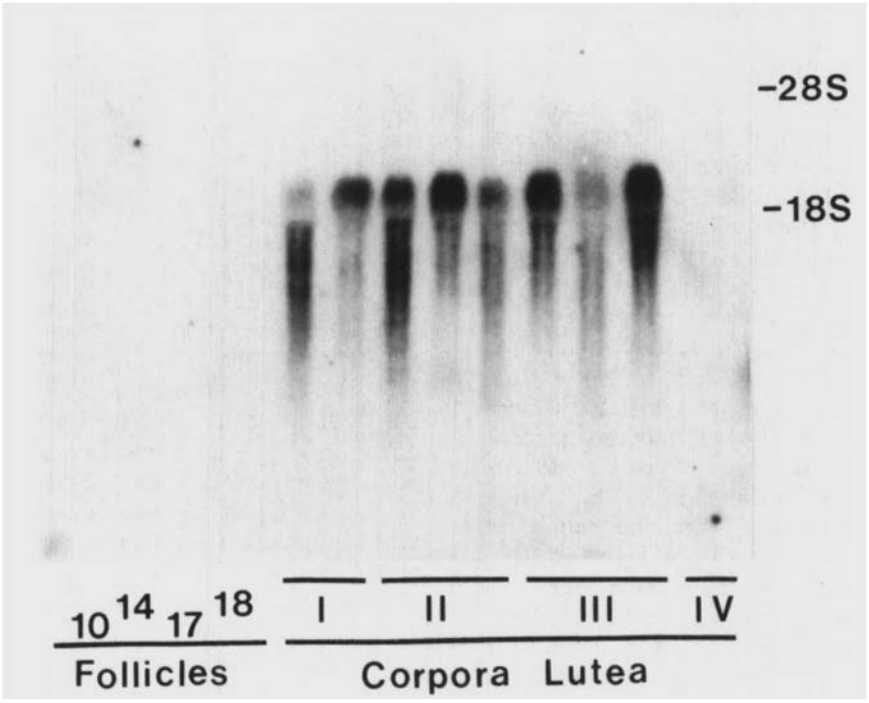

Fig. 1. Relative Levels of P-450_{scc} mRNA in Bovine Follicles and Corpora Lutea as Determined by Northern Blotting Analysis
Hyrbidization to total RNA (20 μg) from individual follicles of differing diameter (10, 14, 17, 18 mm) and individual corpora lutea of differing stages of development (I, II, III, IV) was accomplished as described. Final washing conditions were 15 mM NaCl/1.5 mM sodium citrate at 50 C for 1 h. Positions of 18S and 28S ribosomal RNA are indicated.

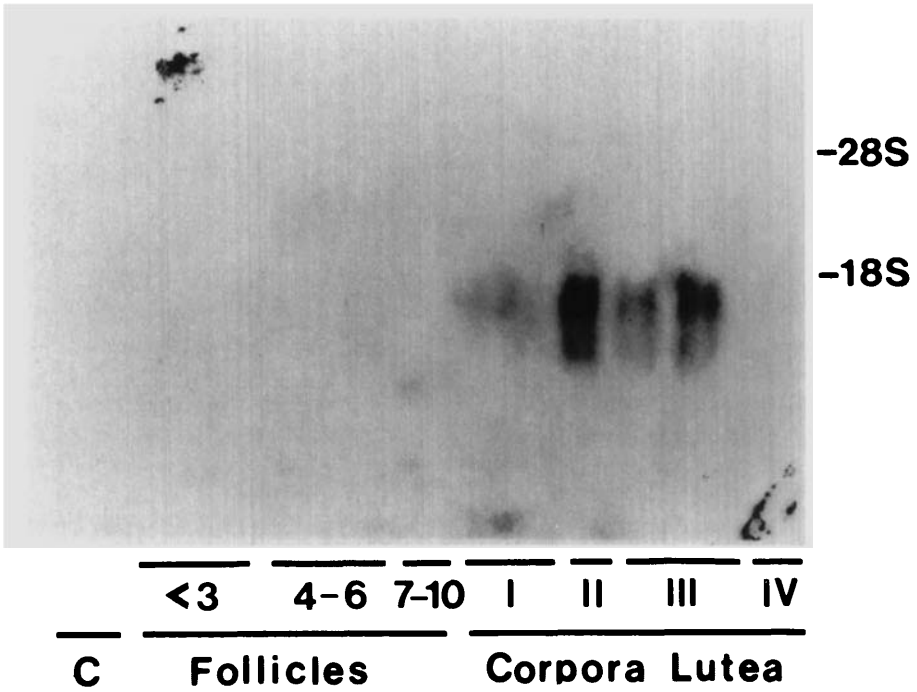

Fig. 2. Relative Levels of Adrenodoxin mRNA in Bovine Follicles and Corpora Lutea as Determined by Northern Blotting Analysis
Hybridization to poly(A)$^+$ RNA (5 μg) from ovarian cortex (c) and pools of follicles of differing diameters (<3 mm, 4–6 mm, 7–10 mm) and individual corpora lutea of differing stages of development (I, II, III, IV) was accomplished as described.

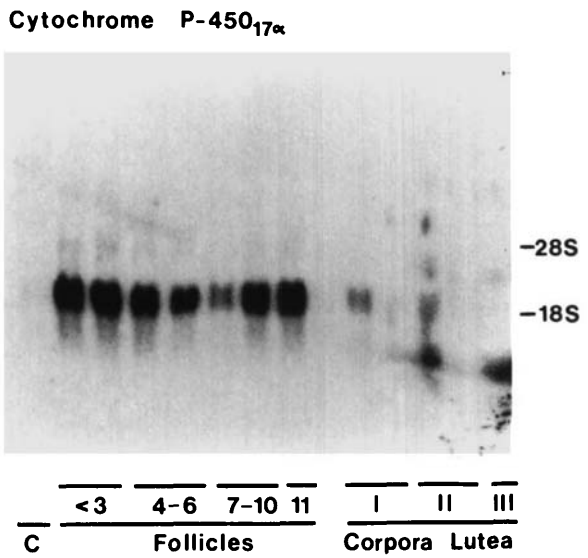

Fig. 3. Relative Levels of P-450$_{17\alpha}$ mRNA in Bovine Follicles and Corpora Lutea as Determined by Northern Blotting Analysis
Hybridization to poly(A)$^+$ RNA (5 μg) from ovarian cortex (c) and individual (11 mm) and pools (<3 mm, 4–6 mm, 7–10 mm) of follicles and individual corpora lutea of differing stages of development (I, II, III) was accomplished as described.

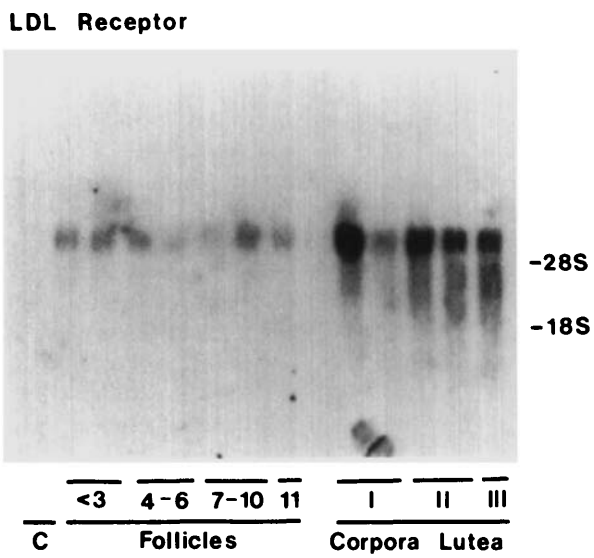

Fig. 4. Relative Levels of LDL Receptor mRNA in Bovine Follicles and Corpora Lutea as Determined by Northern Blotting Analysis
Hybridization to poly(A)$^+$ RNA (5 μg) from ovarian cortex (c), individual (11 mm), or pools (<3 mm, 4–6 mm, 7–10 mm) of follicles of differing diameter and individual corpora lutea of differing stages of development (I, II, III) was accomplished as described.

DISCUSSION

In this study it was found that P-450$_{scc}$ mRNA and adrenodoxin mRNA were present at much higher levels in bovine corpora lutea than in follicles. Conversely P-450$_{17\alpha}$ mRNA was detected only in follicles and young corpora lutea, whereas LDL receptor mRNA was found in both follicles and corpora lutea, but at higher levels in corpora lutea. None of these mRNA species was detected in stage IV, *i.e.* regressionary, corpora lutea.

During the bovine estrous cycle much of the ovarian androstenedione and estrogen is secreted by the larg-

est nonatretic follicle (2). Upon ovulation of the follicle, the rates of synthesis of androstenedione and estrogen decline, and as the corpus luteum develops the rate of synthesis of progesterone increases to an absolute level of steroid hormone production much greater than that of the follicle (2). In a previous study, key steroidogenic enzymes P-450$_{scc}$, adrenodoxin, and P-450$_{17\alpha}$, which catalyze respectively the conversion of cholesterol to pregnenolone and progesterone or pregnenolone into androgens, were detected in follicles by immunoblot analysis, but after ovulation both the total and specific contents of P-450$_{scc}$ and adrenodoxin were substantially greater whereas P-450$_{17\alpha}$ was undetectable (3). By contrast, the specific contents of mitochondrial and microsomal housekeeping enzymes, cytochrome *c* oxidase, and NADPH-cytochrome P-450 reductase, were only minimally altered. Thus, it was suggested that the amount and type of steroid hormone produced is dependent upon the amounts and types of steroidogenic enzymes present in follicles and corpora lutea (3). Further support for this idea was obtained when it was found that the known steroidogenic capacities of the subcompartments of follicles and corpora lutea were related to their content of steroidogenic enzymes, namely that the theca interna, known to produce androgens, stained positively for both P-450$_{scc}$ and P-450$_{17\alpha}$ and that the membrana granulosa, known to be able to produce progesterone but not androgens, stained positively, to a variable degree, for only P-450$_{scc}$ (4). Small and large luteal cells, which are known both to be able to produce progesterone and as mixed populations to produce only relatively little androgen, stained positively for P-450$_{scc}$ but not for P-450$_{17\alpha}$ (4, 5).

In the present study it was shown that during follicular and luteal development the changes in the specific contents of mRNA for P-450$_{scc}$, adrenodoxin, and P-450$_{17\alpha}$ were similar to those observed for their respective proteins. From these results it would appear that the amounts and types of steroid hormones produced are dependent on the levels of the various steroidogenic enzymes (3) which are in turn dependent on the levels of the mRNA species encoding these enzymes.

Steroid hormone production is dependent not only upon the contents of the steroidogenic enzymes but also on the provision of substrate (cholesterol) for the steroidogenic pathway. Cholesterol is derived by *de novo* synthesis within the cell or from the plasma by the uptake of cholesterol-containing lipoproteins (7, 8). As an ovulated follicle develops into a corpus luteum and its content of P-450$_{scc}$ and its rate of progesterone production increase, so too must the demand for substrate. Recently it was shown that the content of HMGCoA reductase, a key regulatory enzyme in the synthesis of cholesterol, was substantially greater in bovine corpora lutea than in follicles (6) suggesting that *de novo* synthesis of cholesterol is an important mechanism by which cholesterol is obtained. Moreover, in that study it was suggested that the regulation of HMGCoA reductase in bovine luteal cells was dependent upon the cellular cholesterol levels as in other cell types, and hence related to the rate of steroidogenesis as a consequence of substrate use. In the present study LDL receptor mRNA was found to be present in both follicles and corpora lutea but higher levels were present in corpora lutea. Taken together, the results from both studies suggest that as follicles develop into corpora lutea the demand for cholesterol as substrate for steroidogenesis, and for cell membrane synthesis, increases and is met by both *de novo* synthesis of cholesterol and the uptake of cholesterol in the form of lipoproteins.

In conclusion, it would appear that the episodic changes in the amounts and types of steroidogenic hormones produced by bovine ovaries are dependent upon the amounts and types of steroidogenic enzymes present. These in turn are dependent upon the levels of mRNAs encoding these enzymes. In parallel, corresponding changes in the levels of proteins involved in provision of cholesterol substrate for progesterone synthesis, namely the LDL receptor and HMGCoA reductase, also occur. Based on our previous conclusions that the increase in HMGCoA reductase of bovine luteal cells by $(Bu)_2$ cAMP was blocked by an inhibitor of cholesterol side-chain cleavage (6), it appears likely that the changes in HMGCoA reductase are secondary to cholesterol use for progesterone biosynthesis. The mechanisms whereby the changes in levels of mRNA encoding steroidogenic enzymes occur, as well as the factors responsible for these changes, remain to be determined.

MATERIALS AND METHODS

Tissues

Bovine ovaries were collected at an abattoir within 20 min of death. Tissues were kept in ice-cold Dulbecco's modified Eagle's medium during dissection of follicles and corpora lutea. Follicles which by visual inspection appeared to be healthy and nonatretic were chosen for this study. It has been shown that in healthy nonatretic follicles the membrana granulosa is intact, whereas that of atretic follicles is disrupted and has degenerated (9, 10). In this study the status of the membrana granulosa was assessed visually using transillumination of the follicle; and where follicles were obviously atretic they were discarded. A range of sizes of antral follicles were collected and subsequently smaller follicles were pooled into groups based upon their diameter. Corpora lutea were collected and classified into four groups by the method of Ireland *et al.* (11) based upon their size, color, presence or absence of blood clots, and other gross morphological features. The four groups were designated as stages I, II, III, and IV and corresponded to the early, early-mid, late-mid, and the regressionary stages of the luteal phase of the estrous cycle respectively (3, 11). Ovarian stroma which did not contain any follicles greater than 0.5 mm was also collected and all tissues were frozen on solid CO_2 and stored at −70 C.

RNA Isolation

RNA was prepared essentially by the method of Ilaria *et al.* (12). Groups of small antral follicles, individual large follicles (>10 mm diameter) and segments of luteal tissue (≃1 g) were homogenized in 5 ml 6 M guanidinium hydrochloride solution

containing 200 mM sodium acetate and 1 mM dithiothreitol using a polytron (30–60 sec). Particulate matter was removed by centrifugation (12,000 × *g*, 15 min, 4 C) and RNA was precipitated from the supernatant by the addition of 2.5 ml ethanol (−20 C, 1 h). The RNA was then centrifuged into a pellet (12,000 × *g*, 15 min, −20 C), the supernatant was discarded, and the RNA was dissolved in 5 ml 6 M guanidinium hydrochloride solution as above. This procedure was repeated twice more and the RNA was dissolved in 1.2 ml Tris-buffered (30 mM, pH, 7.4) sodium chloride solution (100 mM) containing 1 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 400 μg proteinase K/ml and incubated at 37 C for 1 h. This solution was then extracted twice with 1.2 ml phenol-chloroform-isoamyl alcohol (100:100:1) and the RNA precipitated by the addition of ethanol (2.5 ml, −20 C, overnight). The RNA was then centrifuged into a pellet and washed twice with 2 ml 70% ethanol. The ethanol was removed under vacuum and the RNA was resuspended in 0.5 ml H_2O. The mean ratio of optical densities at 260 nm and 280 nm of RNA solutions was 1.7 ± 0.02 (SEM) and the mean yields of RNA per follicle were, respectively, 28, 48, 118, and 391 μg RNA for follicles of diameters less than 3 mm, 4–6 mm, 6–10 mm, and greater than 10 mm. The yield of RNA from all classes of corpora lutea was similar and was 2.2 ± 0.12 mg/g frozen tissue.

Poly(A)$^+$ RNA was isolated from the samples of RNA by affinity chromatography on oligo(dT)-cellulose (Pharmacia P-L. Biochemicals, Uppsala, Sweden). The samples of RNA were first heated (65 C, 15 min), then quenched on ice, and mixed with an equal volume of oligo(dT)-cellulose (20 mg/ml) in 1 M KCl, 2 mM EDTA, 20 mM Tris (pH 7.6). This slurry was continually mixed on a stirrer for 1 h at room temperature and then the oligo(dT)-cellulose was centrifuged into a loose pellet (1000 rpm, 2 min) and the supernatant was discarded. The oligo(dT)-cellulose was then resuspended in 0.5 M KCl, 1 mM EDTA, 10 mM Tris (pH 7.6), and washed a total of three times in this buffer. To separate the poly(A)$^+$ RNA from the oligo(dT)-cellulose, successive rinses of the oligo(dT)-cellulose were carried out with H_2O (4 × 250 μl) and these were collected and pooled. The concentration of RNA was determined by measuring the optical density of the RNA solutions at 260 nm and the mean yield of poly(A)$^+$ RNA was 4.0 ± 0.3% of the total RNA. The mean ratio of optical densities at 260 nm and 280 nm of solutions of poly(A)$^+$ RNA was 2.1 ± 0.01.

Northern RNA Blotting

RNA samples were subjected to electrophoresis through agarose gels (0.8 or 1.25%) containing formaldehyde (6%) using a 20 mM morpholinopropanesulfonic acid buffer (pH 7.0) containing 5 mM sodium acetate and 0.1 mM EDTA (13). The RNA was then electroblotted (30 V, O/N or 80 V, 4 h) to ζ-probe (Bio-Rad Laboratories, Richmond, CA) in 10 mM Tris (base), 5 mM sodium acetate, and 0.5 mM EDTA. The blots were then baked (80 C, 2 h) and exposed to ultraviolet radiation for 5 min. Prehybridization was carried out in a solution containing 10× concentrated Denhart's solution, 0.6 M NaCl, 60 mM sodium citrate, 1 mM EDTA, 0.5% SDS, 100 μg sonicated, denatured salmon sperm DNA/ml and 10 μg polyadenylic acid/ml (14) at 65 C overnight. Hybridizations were carried out under the same conditions. The blots were then washed at room temperature in 0.45 M NaCl/45 mM sodium citrate/1 mM EDTA/0.1% or 1.0% SDS (three times, 20 min each) and then at 50 C at repeatedly lower salt concentrations (0.3 M NaCl/30 mM sodium citrate, 0.15 M NaCl/15 mM sodium citrate, 75 mM NaCl/7.5 mM sodium citrate, 15 mM NaCl/1.5 mM sodium citrate). Blots were then air-dried and subjected to autoradiography using intensifying screens at −70 C and exposure times up to 18 h.

cDNA Probes

Cytochrome P-450$_{scc}$ Plasmid pBSCC-2 (14), which has an insert with a sequence specific for approximately or equal to 650 base pairs of the 3′-end of the coding region of mRNA for P-450$_{scc}$ from bovine adrenal cortex (15), was labeled by nick-translation using ^{32}P-deoxycytidine-5′-triphosphate (dCTP) and other materials in a kit from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, MD). The labeled probe was purified by centrifugation through a minicolumn of Bio-Gel P-30 in an apparatus similar to that used for desalting small amounts of protein (16). The specific activity was greater than 10^8 cpm/μg DNA.

Adrenodoxin Plasmid pBAdx4 (17), which contains a 900-base pair sequence coding for the entire amino acid sequence of bovine adrenal cortical adrenodoxin, was digested with restriction endonuclease *Bam*HI and the insert containing the entire coding sequence purified by electrophoresis on an agarose gel followed by electroelution. Labeled hybridization probes were prepared by synthesizing complementary strands of DNA using the Klenow fragment of DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN), and random hexamers of single stranded DNA as primers (18) and [^{32}P] dCTP (New England Nuclear, Boston, MA). The ^{32}P incorporated into DNA was separated from the free ^{32}P by the same method as described above. The specific activity of DNA labeled by this method has been reported to be greater than 10^9 cpm/μg DNA (18).

Cytochrome P-450$_{17\alpha}$ Plasmid pcD17α-2 (19), which contains a sequence coding for the complete amino acid sequence of bovine adrenal cortical P-450$_{17\alpha}$, was digested with restriction endonuclease *Pst*I and a fragment coding for almost the full-length of the mRNA (19) was purified by electrophoresis on an agarose gel. The fragment was removed from the gel by electrophoresis on to an NA-45 membrane filter from which the DNA was eluted in 1 M NaCl, 1 mM EDTA, and 20 mM Tris (pH 8.0) at 60 C. Radiolabeled cDNA was prepared by the same method as used for adrenodoxin.

LDL Receptor Three single-stranded DNA M13 vectors containing inserts complementary to three segments of the cDNA of the coding and noncoding regions of the mRNA for bovine LDL receptor were obtained from Dr. David Russell of this Institution. These inserts (a, b, and c) had been obtained by restriction enzyme digests of the cDNA with (a) *Pst*I, (b) *Xba* and *Xho*I, and (c) *Hind*III and *Xba* (20-22 for restriction enzyme maps and base sequence). A second strand of DNA was synthesized using the Klenow fragment of DNA polymerase and [α^{32}-P]dCTP and the universal primer of M13 (23). The newly synthesized radiolabeled single strands of DNA (≃100 base pairs), which were complementary to the RNA encoding the LDL receptor, were separated from the large M13 strands by electrophoresis on a polyacrylamide-urea gel (23) using autoradiography to locate the radiolabeled DNA. The three radiolabeled single stranded cDNAs were then pooled for hybridization.

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