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MOLECULAR CLONING OF DNA COMPLEMENTARY TO BOVINE ADRENAL $P450_{scc}$ mRNA

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SUMMARY: $P450_{scc}$ is the rate-limiting hormonally regulated enzyme that cleaves the cholesterol side chain. Translation of bovine adrenocortical mRNA and immunoprecipitation with rabbit anti-bovine $P450_{scc}$ indicates $P450_{scc}$ mRNA represents 1% of the total. DNA complementary to bovine adrenocortical mRNA was cloned in the PstI site of pBR322 by dC·dG tailing and high-efficiency transformation. A clone containing sequences complementary to $P450_{scc}$ mRNA was identified by hybrid-selected translation only when plasmid DNA was first purified by CsCl gradient centrifugation. As is often the case with hybrid-selected translation, the clone identified contains a small insert.

The first step in the synthesis of steroid hormones *in vivo* is the conversion of cholesterol to pregnenolone by the cleavage of the cholesterol side chain. This step, classically called "20,22 desmolase," is rate-limiting (1) and regulated in the adrenal fasciculata by ACTH (2,3). It may also be regulated by angiotensin II in the adrenal glomerulosa, and by leutinizing hormone in the gonad. Supraphysiologic concentrations of ACTH increase synthesis of $P450_{scc}$ mRNA only 4- to 9-fold above normal (4,5), indicating ACTH regulates $P450_{scc}$ gene transcription. To study this regulation in greater detail and to study disease states due to defective activity of this enzyme, we have cloned cDNA for bovine $P450_{scc}$.

MATERIAL AND METHODS

Preparation and translation of RNA. Polyadenlyated RNA ($poly(A)^+$ RNA) from fresh bovine adrenal cortices was prepared and translated as described (6). Translation products were immunoprecipitated with rabbit anti-bovine $P450_{scc}$ (4) and analyzed on SDS/polyacrylamide gels as described (6).

Construction of the bovine adrenal cDNA library. $Poly(A)^+$ RNA was reverse transcribed twice into double-stranded cDNA with the addition of 1mM RNasin (Promega Biotec, Madison WI) during the first reverse transcription.

Abbreviations: ACTH, adrenocorticotrophic hormone; bp, base pairs; kb, kilobases; dC, deoxycytidine; dG, deoxyguanosine; SDS, sodium dodecyl sulfate.

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The double-stranded cDNA was trimmed with DNA S_1 nuclease, and the 3' ends were extended with deoxycytidine as described (7,8). The tailed cDNA was then size-selected by electrophoresis through low-gelling temperature ("soft") agarose. cDNA of <500 bp was discarded and the remainder was handled in two batches of 500-1000 bp, and 1000-4000 bp. These two batches respectively contained about 55% and 18% of the total radioactivity initially loaded on the gel. This size-selected cDNA was then inserted into the PstI site of pBR322 which had been previously tailed with deoxyguanosine residues. The recombinant plasmids were then used to transform E. coli MM294 using a high-efficiency transformation system (9).

Hybrid-selected translation. Groups of 4 to 9 clones were grown with an additional clone of bovine growth hormone cDNA (8) in 150ml cultures in minimal medium with chloramphenicol amplication. Plasmid DNA was prepared by polyethylene glycol precipitation from a cleared lysate, cleaved with EcoRI or BamHI to linearize the plasmid, denatured, and affixed to 2.5cm nitrocellulose filters as described (10-12). Filters were hybridized at 42° in 50% formamide overnight with 40µg of bovine adrenocortical mRNA and 3µg of bovine pituitary mRNA. The filters were washed extensively with 1 x SSC/50% formamide and the specifically bound mRNAs were eluted with water and precipitated with ethanol. This RNA was translated, the translation products were immunoprecipitated, and the immune complexes were displayed on 12.5% SDS/polyacrylamide gels and analyzed by fluorography for 2 to 4 weeks. The bovine growth hormone plasmid in conjunction with the bovine pituitary mRNA (12% bovine growth hormone) provide an internal control for each hybrid-selected translation.

RESULTS

Preparation and translation of RNA. The bovine adrenocortical poly(A)$^+$ RNA supported efficient translation and encoded a wide array of proteins seen by incorporation of [^{35}S]methionine and autoradiography after SDS/polyacrylamide gel electrophoresis (Fig. 1). Immunoprecipitation of this material with rabbit anti-bovine $P450_{scc}$ (4) produced a single band having an apparent molecular weight of 55,000, in good agreement with the 54,500 dalton figure previously published for bovine pre-$P450_{scc}$ (4,5). When excess $P450_{scc}$ antigen was added exogenously, this band could not be immunoprecipitated (not shown). As estimated by specific incorporation of radioactivity and by densitometric scanning of the gel (13) we estimate the mRNA for $P450_{scc}$ represents approximately 1% of total bovine adrenocortical mRNA.

Construction of the bovine adrenal cDNA library. Transformations using the Hanahan protocol (9) in E. coli produced about 5 x 10^7 to 10^8 transformants per microgram of supercoiled pBR322 and 400 to 800 recombinant transformants per nanogram of input tailed cDNA, approximately 100-fold better than previous procedures (7,8). Several transformations yielded about 10,000 clones from cDNA of 1000-4000 bases and 50,000 clones from the cDNA of

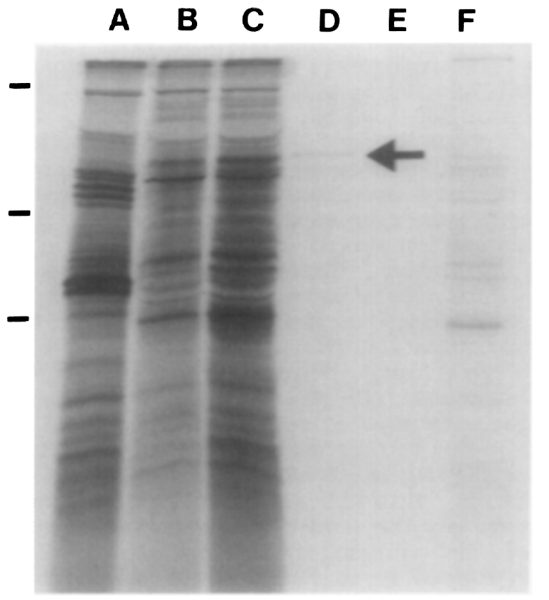

Figure 1. Cell-free translation and immunoprecipitation. Lane A: Translation of control RNA from an ectopic ACTH-producing tumor (14). Lanes B and C: Translation of two different preparations of bovine adrenocortical polyadenylated RNA. Lane D: Immunoprecipitation using rabbit anti-bovine $P450_{scc}$. The arrow indicates the specifically immunoprecipitated pre-$P450_{scc}$. Lane E: Non-radioactive molecular weight markers seen by staining prior to autoradiography. Lane F: Aliquot of supernatant following immunoprecipitation with excess anti-$P450_{scc}$; note the presence of other bands of the same molecular weight as pre-$P450_{scc}$ which remain. Molecular weights indicated by the marks to the left of Lane A: 80, 42, and 25 thousand daltons.

500-1000 bases. By antibiotic resistance criteria, >95% of the transformants contain recombinant plasmids. The sizes of the cloned cDNA inserts from ten random clones from the large library (1000-4000 bp) were 800-3400 bp and from ten random clones from the small library (500-1000 bp) were 400-900 bp.

Hybrid-selected translation. We use hybrid-selected translation rather than the simpler hybrid-arrested translation procedure (15,16) because cell-free translation of bovine adrenal mRNA yields other proteins having essentially the same electrophoretic mobility as $P450_{scc}$ on an SDS/ polyacrylamide gel (Fig. 1, lane F); hence, the lack of $P450_{scc}$ band would not be detectable. We intially screened pools of nine unknown clones plus the bovine growth hormone clone with the plasmid DNAs prepared by polyethylene glycol precipitation from a cleared lysate. After screening about 500 clones without finding a $P450_{scc}$ cDNA, we reduced the number of

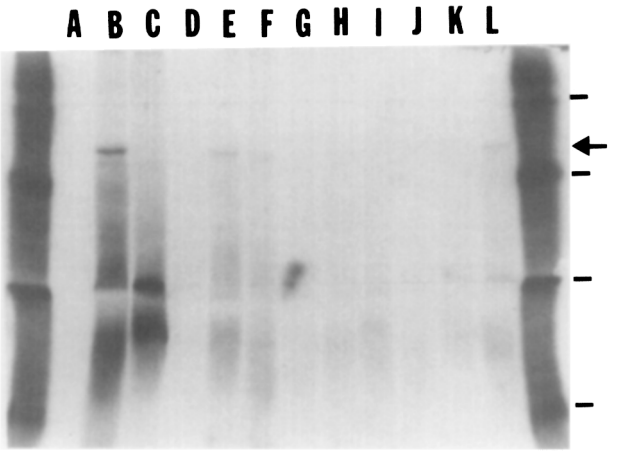

Figure 2. Identification of two pools of clones containing bovine $P450_{scc}$ cDNA. Lanes A and B: Translation products of bovine adrenal mRNA immunoprecipitated with anti-ovine growth hormone (A) and anti-bovine $P450_{scc}$ (B). Lanes C to L: Translation and anti-$P450_{scc}$ immunoprecipitation products of bovine adrenal mRNA selected by hybridization to plasmid DNA isolated from pools of 4 clones each. The outer lanes contain molecular weight markers of 92, 69, 46 and 30 thousand daltons. The arrow indicates the migration of authentic bovine $P450_{scc}$. This gel was fluorographed in DMSO/PPO; hence, the resolution is poorer than the autoradiograph in Figure 1.

unknown clones in each pool to four (plus bovine growth hormone) and prepared all the plasmid DNAs by CsCl centrifugation. These altered procedures quickly led to the identification of two positive pools of clones (Fig. 2). The pools in lanes E and L were deemed positive because of the presence of an immunoprecipitated band that co-migrates with authentic, immunoprecipitated bovine $P450_{scc}$. The band in lane F appears to be a false-positive as its migration in the gel is faster than authentic $P450_{scc}$. The pool of clones in lane L was chosen for further study. When these were examined individually, three of the four clones hybrid-selected an mRNA encoding a protein which immunoprecipitates with anti-bovine $P450_{scc}$ (Fig. 3). Restriction endonuclease mapping of the clones in lanes B, C, D and E shows that the clones in D and E contain identical 230 bp inserts. This suggests one of these was a "satellite colony" of the other; these clones are designated pBA571 (plasmid bovine adrenal). Lane B (pBA574) and lane C (pBA573) contain cloned inserts of 150 and 1700 bp, respectively. The cloned cDNA of pBA571 does not hybridize to the cloned cDNA of pBA573 or 574 on Southern blots. Since pBA571 and pBA574 do not cross-hybridize, they probably arose from

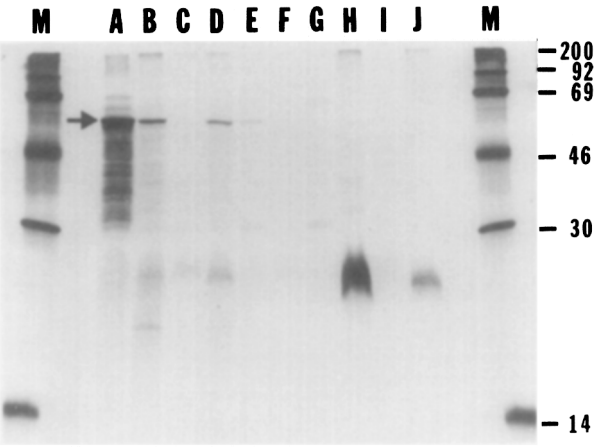

Figure 3. Identification of bovine $P450_{scc}$ cDNA clones by hybrid-selected translation. Lane A: Translation products of bovine adrenal mRNA immunoprecipitated with anti-$P450_{scc}$ (arrow). Lanes B, C, D and E: Translation products immunoprecipitated with anti-bovine $P450_{scc}$ from mRNA selected by the individual clones in the positive pool, each prepared by CsCl gradient ultracentrifugation of plasmid DNA. Lane F: Hybrid selection of the clones in lanes B and C. Lane G: Hybrid-selection of the clones in lanes D and E, each prepared by polyethylene glycol precipitation from a cleared lysate. Lane H: Translation products of bovine pituitary mRNA immunoprecipitated with anti-ovine growth hormone antibody (smear at about 24-25,000 daltons). Lanes I and J: Translation products of bovine pituitary mRNA selected by our bovine growth hormone cDNA plasmid and immunoprecipitated with anti-ovine growth hormone. In lane I the bovine growth hormone was precipitated from cleared lysate with polyethylene glycol and in lane J it was prepared by CsCl gradient centrifugation. Lane M: Molecular weight markers (sizes indicated at right in thousands of daltons).

different regions of the mRNA. The discrepancies between lanes I versus J, B and C versus F, and D and E versus G in Figure 3 indicate that CsCl-purified plasmid DNA is much more effective in hybrid-selected translation. The bovine growth hormone plasmid, bovine pituitary mRNA, and anti-ovine growth hormone antibody were included in lanes B, C, D and E; successful hybrid selection of this internal control is seen in each lane.

DISCUSSION

Hybrid-selected translation is frequently the only means available to identify a cDNA clone representing an mRNA of low abundance. Enrichment of the abundance of the $P450_{scc}$ mRNA was not possible in cattle, and when the experiment was done with 15-day weanling or adult rats the apparent enrichment of $P450_{scc}$ mRNA was from a control of 0.8 to a stimulated level of 1.3% (W.L. Miller, unpublished observations). Size selection of mRNA, either on gels or sucrose gradients, rarely yields greater than 3-fold enrichment

when the mRNA is similar in size to the great bulk of cellular mRNAs (∿2 kb). We size-selected our double-stranded cDNA before cloning, expecting P450 cDNAs to be enriched in the fraction >1 kb and diminished in the smaller fraction. Identification of very small cDNAs from the library of large cDNA clones was a surprising finding. Our previous experience (K.J. Matteson, unpublished) and that of many others (12,17,18, M.R. Waterman, personal communication) is that small clones have been identified. Since the cDNA clones we screened were constructed from cDNA of $\geq$ 1 kb, and since all of the dozens of other clones which we have examined from this library contained cDNAs larger than 800 bp, we suggest that small cDNAs may be preferable for identification by hybrid selection. Although all cDNAs should select the corresponding mRNA and all such heteroduplexes should be liberated by heating to 90°C in water, it appears that a small region of hybridization results in more efficient release of translatable mRNA from the cDNA filter.

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