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THE PARTIAL PURIFICATION OF RABBIT LIVER CYTOCHROME P450-LM_2-mRNA

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SUMMARY. We have partially purified the mRNA coding for the phenobarbital induced rabbit liver cytochrome P450-LM_2 approximately 90 fold relative to control liver levels. Using a combination of Sepharose 4B chromatography, denaturing and non-denaturing sucrose gradient centrifugation, we have obtained a mRNA that when translated *in vitro* using wheat germ lysate codes for 7.2% cytochrome P450-LM_2.

INTRODUCTION. The role and function of the liver cytochrome P450's in the metabolism and detoxication of both endogenous and exogenous compounds has been studied for a considerable number of years. However, studies on the regulation of the synthesis of these proteins has been hampered by the lack of a suitable assay for identifying the specific cytochrome P450-mRNAs. Recently, there have been several reports demonstrating that specific cytochrome P450-mRNAs can now be identified using a combination of *in vitro* translation followed by an immunoprecipitation assay using a suitable antibody (1-6). The levels of the specific translatable cytochrome P450-mRNA's in phenobarbital induced rat liver range from 0.20-4% (4,2), and for rabbit liver, 0.63% (6). Studies are also being carried out using mouse liver (7), and bovine adrenals (8). Further progress in studying these mRNA's requires the extensive purification of the mRNAs or the isolation of specific sequences using recombinant DNA technology.

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Using a combination of sizing procedures, we have obtained a rabbit liver mRNA fraction that is significantly enriched for the phenobarbital induced cytochrome P450-LM_2-mRNA (LM_2-mRNA). This purification process should have a general application to the study of other cytochrome P450-mRNAs.

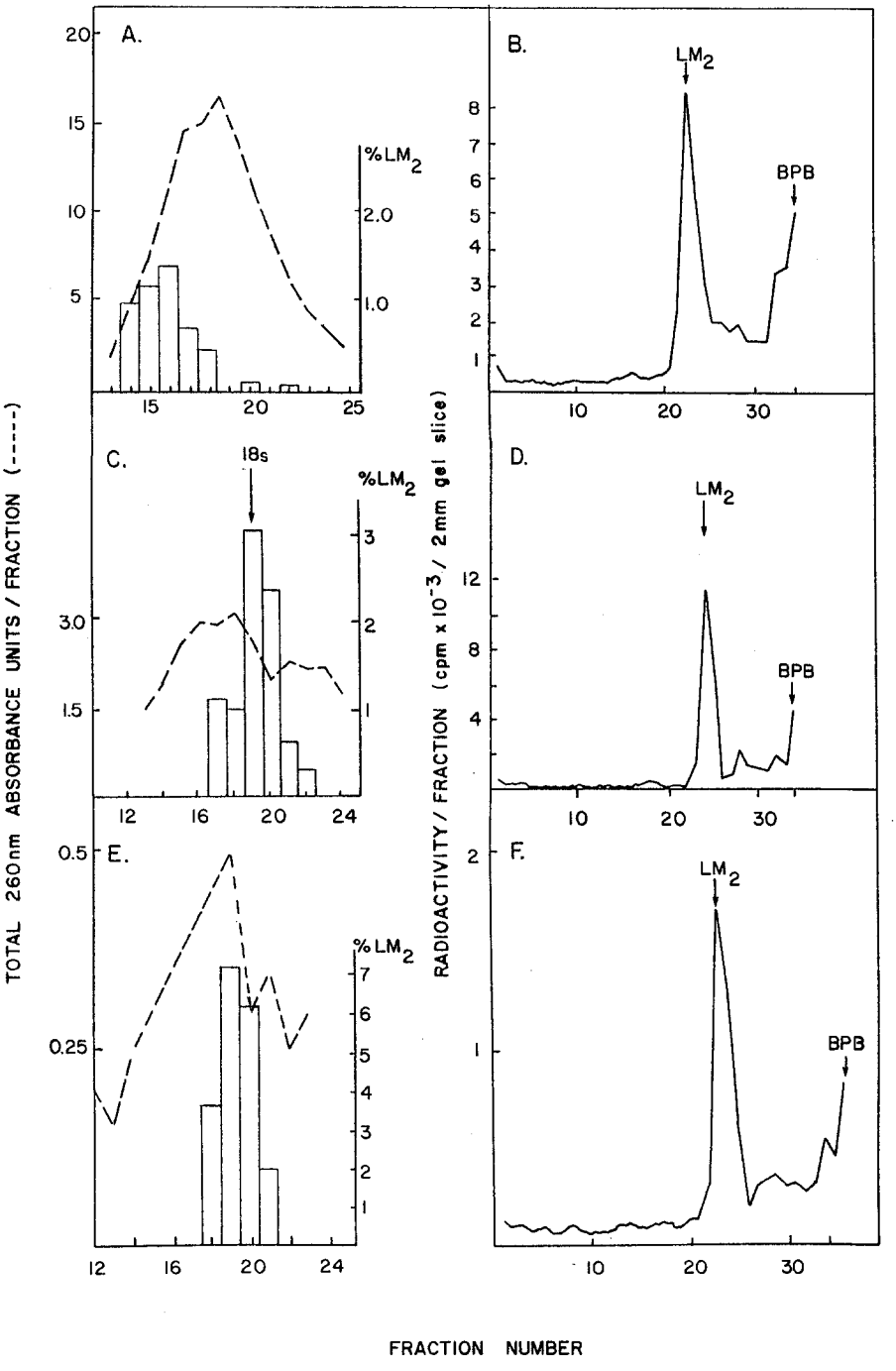
MATERIALS AND METHODS

Male rabbits (NZ Whites, 2-2.5 kg) were obtained from a local rabbitry and kept under standard vivarium conditions. Phenobarbital was injected i.p. (80 mg/kg bw) every 24 hrs and the animals killed 12hr after the second injection. The livers were rapidly removed, trimmed free of connective tissue and gall bladder, and either used immediately for the preparation of polysomes (9), or stored at -70°C until use. Polysomal RNA was isolated by phenol-$CHCl_3$ extraction (8) and recovered by ethanol precipitation. After washing with 2M LiCl (9), the polysomal RNA was chromatographed through an oligo(dT) cellulose column to separate poly(A+)mRNA from rRNA and poly(A-)mRNA (10). Translation of the poly(A+)mRNA was carried out using wheat germ lysate and [^{3}H] leucine as described (6,11). The in vitro synthesis of cytochrome P450-LM_2 was quantitated as follows. The translation reactions were centrifuged at 100,000xg for 1 hr, and the ribosome free supernatant brought to 0.5% Triton X-100, 0.5% sodium deoxycholate, 10mM $NaPO_4$ (pH 7.4), 10mM leucine, and 0.15M NaCl. Carrier cytochrome P450-LM_2 (LM_2, 15 µg) was added, followed by 20 µl of goat-anti-LM_2 IgG (6). After incubation at room temperature for 1 hr, the samples were transferred to 4° for 16 hr. The immunoprecipitate was collected by centrifugation, washed twice with phosphate buffered saline containing 10mM leucine, and then heated at 90° in 200 µl of 3% sodium dodecylsulphate (SDS), 10% glycerol, 5% mercaptoethanol, 60mM Tris-HCl, pH 6.8, until it dissolved. The cooled supernatant was analyzed by SDS-7.5% polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were sliced into 2mm pieces and the [^{3}H] content of each piece determined as described previously (6).

Sepharose 4B chromatography of liver poly(A+)mRNA was carried out as described (12). Fractions (5ml) were collected from the column (1.6 x 100 cm), and the mRNA in each fraction recovered by ethanol precipitation. The recovered mRNA was dissolved in 50-100 µl of sterile distilled water, and the RNA concentration determined by absorption at 260nm ($1A_{260}$ = 40 µg RNA). Aliquots of each fraction were translated in 200-500 µl reactions (2 µg mRNA/50 µl reaction), and in vitro LM_2 synthesis quantitated as described earlier (6). The column fractions which contained the LM_2-mRNA were combined, brought to 5mM Tris-HCl, pH 7.4, 0.5mM EDTA, 0.2% SDS, heated, quick cooled, then layered over 10-35% linear sucrose gradients (400 µg mRNA/12 ml gradient) containing the same buffer (13). After centrifugation at 40,000 rpm for 16 hr at 20°, 0.5 ml fractions were collected, and the mRNA recovered and quantitated. The mRNA fractions from three gradients were combined and analyzed for LM_2-mRNA as before. The LM_2-mRNA containing fractions were combined, adjusted to 100mM NaCl, 1mM

EDTA, 10mM Tris-HCl pH 7.5, heated, cooled, then analyzed using 5-20% linear sucrose gradients (14) by centrifugation at 40,000 rpm at 2° for 9 hr. The mRNA was recovered, quantitated and translated as before, except 100-200 µl reactions were used for the in vitro analysis of the LM_2-mRNA. Rabbit albumin mRNA was assayed by carrying out the immunoprecipitation with 15 µg albumin, 20 µl goat antirabbit albumin IgG, followed by 7.5% SDS-PAGE as described (6).

RESULTS AND DISCUSSION. We have previously shown that phenobarbital causes a dramatic increase in liver protein synthesis 12 hr after the administration of phenobarbital by injection (15). Peak translatable LM_2-mRNA levels were found 12 hr after the second phenobarbital injection (data not shown) and represents 0.25-0.35% of total liver polysomal poly(A+)mRNA driven in vitro protein synthesis. This represents a 3-5 fold increase over untreated rabbit liver levels (i.e. 0.05-0.08%). Using liver polysomal poly(A+)mRNA from a 36hr phenobarbital treated rabbit (i.e., killed 12 hr after the second injection of phenobarbital), we loaded 8mg mRNA (average 0.30% LM_2) onto the Sepharose 4B column. The analysis of the eluate is shown in Fig. 1A. It can be seen that the LM_2-mRNA is found in fractions 14-17 and is separated from much of the liver poly(A+)mRNA (solid line). The 28s rRNA is found in fractions 29-32, and DNA (if present) in fractions 7-10 (data not shown). The SDS-PAGE analysis of the goat-anti-LM_2 IgG immunoprecipitate obtained from translating the mRNA in fraction 16, is shown in Fig. 1B. Apart from some low molecular weight material at the bottom of the gel, all the radioactivity comigrates with authentic cytochrome P450-LM_2. Fractions 14-17 from the sepharose 4B column were combined (1.25mg mRNA, average 1.1% LM_2) and analyzed by centrifugation on 10-35% linear sucrose gradients. The gradient fractions were analyzed for LM_2-mRNA, and the results are shown in Fig. 1C. The peak LM_2-mRNA fraction (No 19) represents 3.0% in vitro LM_2 synthesis, comigrates with a


A.
B.
C.
D.
E.
F.
LM2
BPB
18s
%LM2
TOTAL 260nm ABSORBANCE UNITS / FRACTION (-----)
RADIOACTIVITY / FRACTION (cpm x 10-3 / 2mm gel slice)
FRACTION NUMBER

18s rRNA marker (in a parallel gradient) and is separated from much of the liver mRNA (solid line). Peak levels of translatable albumin mRNA were found in fraction 18 (data not shown). This indicates that LM_2-mRNA has a greater sedimentation coefficient than albumin mRNA and therefore could if necessary be separated from albumin mRNA. The SDS-PAGE analysis of the goat-anti-LM_2 IgG immunoprecipitate from the translation of the mRNA in fraction 18 is shown in Fig. 1D. Once again all the radioactivity in the gel comigrates with authentic LM_2. Fractions 19-20 from the 10-35% sucrose gradient were combined (130 μg mRNA, average 2.7% LM_2) and analyzed by centrifugation through a 5-20% linear sucrose gradient. The analysis of this gradient is shown in Fig. 1E, and in fraction 19, *in vitro* LM_2 synthesis represents 7.2% of total protein synthesis. The SDS-PAGE analysis of this fraction is shown in Fig. 1F. This represents a 90 fold enrichment over control, and a 24 fold enrichment over phenobarbital induced liver levels. It should be noted that the value of 7.2% may be an underestimate of actual *in vitro* LM_2 synthesis, because we

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Figure 1. Partial purification of LM_2-mRNA by Sepharose 4B chromatography and sucrose gradient centrifugations.

A. Analysis of Sepharose 4B column fractions. mRNA was quantitated by absorbance at 260nm (----), *in vitro* LM_2 synthesis (shown as a histogram) was determined as described (see materials and methods). Peak *in vitro* LM_2 synthesis is in fraction 16.
B. SDS-PAGE profile of the immunoprecipitate obtained from the analysis of fraction 16 from A. (the total released peptides contained 1,228,545 cpm).
C. Fractions 14-17 from A were combined and analysed on 10-35% sucrose gradients. The three gradients were fractionated, the mRNA recovered, and the corresponding mRNA fractions combined and analysed (see Materials and Methods). Peak *in vitro* LM_2 synthesis is in fraction 19.
D. SDS-PAGE profile of the immunoprecipitate obtained from the analysis of fraction 19 from C (the total released peptides contained 667,600 cpm).
E. 5-20% sucrose gradient analysis of fractions 19-20 from C. Peak *in vitro* LM_2 synthesis is in fraction 19.
F. SDS-PAGE profile of the immunoprecipitate obtained from the analysis of fraction 19 from E. (the total released peptides contained 50,000 cpm) (BPB = bromophenol blue).

have not taken into account the contribution of any shorter peptides resulting from premature termination of translation (16). These peptides could possibly migrate with the tracking dye on SDS-PAGE (Fig. B, D, E). Also, the quantitation of total protein synthesis driven by liver mRNA might be an overestimate because we do not consider the contribution from the endogenous wheat germ mRNA (16) which is a minimum of 6-7%, since liver mRNA driven in vitro protein synthesis is usually 15x background. Previously Sepharose 4B chromatography in conjunction with sucrose gradients has been used for the partial purification of avidin (17) and ovalbumin mRNAs (18), whereas sucrose gradient centrifugation alone has been used successfully to obtain 7-20 fold purifications of corticosteroid binding globulin and phosphenolpyruvate carboxykinase(GTP)mRNAs respectively (13,16). However, this report is the first detailed study of the purification of a liver cytochrome P450 mRNA.

Our data suggests that cytochrome P450-LM_2 mRNA has a similar (or greater) size to that of albumin mRNA, however, LM_2 is a much smaller protein (49,000 vs. 68,000 daltons). Albumin mRNA is estimated to contain 2200-2400 nucleotides (19), and LM_2-mRNA is of similar size, however, only 1278 nucleotides would be required to code for the 424 amino acids in LM_2 (20). In the absence of evidence that cytochrome P450-LM_2 is synthesized as a larger precursor protein (6), we conclude that the mRNA for cytochrome P450 contains large 5' and 3' untranslated nucleotide sequences. The highly enriched mRNA fraction that we have obtained should serve an an excellent template for cDNA and dsDNA synthesis, thus, enabling recombinant DNA technology to be applied to the study of the regulation of cytochrome P450-LM_2 synthesis in normal, induced or diseased liver states.

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