The Upstream Open Reading Frame Mediates Constitutive Effects on Translation of Cytochrome P-450c27 from the Seventh In-frame AUG Codon in Rat Liver*

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The 2.3-kb mRNA that codes for cytochrome P-450c27 (CYP27) has an unexpectedly long 5′-untranslated region (UTR) that holds six AUGs, leading to several upstream open reading frames (uORFs). The initiation of translation from the seventh AUG forms a putative 55-kDa precursor, which is processed in mitochondria to form a 52-kDa mature protein. The first three AUGs form fully overlapping uORF1, uORF2, and uORF3 that are in-frame with the seventh AUG and next two form fully overlapping uORF4 and uORF5 that are out-of-frame with the seventh AUG. Although not recognized by the scanning ribosomes under normal conditions, the sixth in-frame AUG forms a putative 57-kDa extension of the main open reading frame. The purpose of this study was to identify the elements in the 5′-UTR that direct CYP27 mRNA translation exclusively from the seventh AUG. Expression of 5′ deletion mutants in COS cells reveal that the intact 5′-UTR not only directs the initiation of translation from the seventh AUG but also acts as a negative regulator. A 2-kb deletion mutant that lacks uORF1 initiates translation equally from the sixth and the seventh AUGs, forming both 57- and 55-kDa precursor proteins with a 2-fold increase in rate of translation. However, induction in translation does not affect the levels of the mature 52-kDa form in mitochondria but causes accumulation of the precursor form in cytosol not seen in COS cells transfected with wild-type cDNA. Mutation of the stop codon that terminates uORF1 completely shifts the initiation of translation from the seventh to the first AUG, forming a 67-kDa precursor that is processed into a 52-kDa mature protein in mitochondria. Confirmation of the bicistronic nature of CYP27 mRNA by epitope mapping of uORF1 suggests that translation of CYP27 mRNA from the seventh AUG is directed and regulated by uORF1 expression.

Cytochrome P-450c27 (CYP27),1 or sterol 27-hydroxylase, a product of the CYP27 gene, shows a broad tissue and organ distribution and hydroxylates a spectrum of sterol substrates as well as vitamin D(_3)(1–4). CYP27 is located in the mitochondria and catalyzes the initial step in the degradation of the steroid side chain, leading to bile acid biosynthesis in the liver. The 27-hydroxylation of cholesterol is a rate-limiting step in the catabolic conversion of cholesterol to bile acids, and 27-hydroxycholesterol is a regulatory oxysterol that indirectly modulates cholesterol synthesis in the liver and other tissues (5–7). Extrahaepatic CYP27 also converts cholesterol into 27-hydroxycholesterol or 3-hydroxy-5-cholestanolic acid (8, 9), which are then transported to the liver and converted into bile acids. Similarly, CYP27 catalyzes 25-hydroxylation of vitamin D(_3) in the hepatic mitochondria, a first step in the conversion of inactive vitamin D(_3) to the 1α,25-dihydroxy derivative, which is an active hormonal form (10–12). Vitamin D(_3), hormone, a known regulator of mineral metabolism and calcium homeostasis, has more recently been implicated in a number of other biological activities not yet fully understood but clearly related to immunological responses, cell proliferation, and differentiation (11–14). In other minor pathways, extrahaepatic CYP27 also catalyzes 1α-hydroxylation and (24R)-hydroxylation steps in the kidney.

Previously published results report two distinct mRNAs in the rat that code for the synthesis of a similarly sized 55-kDa CYP27 precursor protein (1, 15). The larger 2.3-kb mRNA is predominant but identical to the 1.9-kb mRNA except for a 400-nucleotide 5′-untranslated region (5′-UTR) extension. This 400-nucleotide extension exhibits several unique characteristics, as shown in Fig. 1, which include a polyadenylation signal near the 5′-end; six upstream AUG codons, with the first five forming five upstream open reading frames (uORFs) up to 105 nt long and the sixth AUG initiating a 57-kDa extension of the main open reading frame (ORF); two stem loop structures between nt 184 and 204 and nt 357 and 375; and 100% sequence complementarity between the terminal 290 nucleotides of 2.3-kb mRNA and the predicted translation of the terminal 290 nucleotides of the 5′-translated region of the serine protease inhibitor (SPI-2) mRNA (15–17). The sequence overlap between CYP27 and SPI-2 mRNAs is unique and probably represents the first observation of 5′-end overlap between two functional mRNAs. The formation of RNA duplexes between complementary RNA sequences has been reported in several cell systems (18–20). In the same way, the existence of an RNA duplex formed between the complementary sequences of CYP27 and SPI mRNAs in liver and COS cells was previously demonstrated and shown to have a regulatory role in CYP27 mRNA translation (21). However, the extent of inhibition of translation...
tion did not measure up to the extent to which CYP27 mRNA was involved in duplex formation, suggesting that processing of the RNA duplex is taking place to minimize the inhibitory effect on translation. Furthermore, the presence of a long 5’-UTR in 2.3-kb CYP27 mRNA suggests that complex regulatory mechanisms are involved in the synthesis of CYP27 similar to other known regulatory proteins such as growth factors, cytokines, receptors, and protooncogenes (22, 23).

Mullick et al. (24) reported isolation of the rat CYP27 gene comprising 11 exons encoding the entire 2.3-kb mRNA. They identified and characterized a distinct transcription promoter within the 5’-terminal region of exon 2 that was shown to drive the expression of the shorter 2-kb mRNA, suggesting that the two mRNAs that represent CYP27 in rat liver are products of the same gene locus but have two independent promoter regions. They identified an immediate upstream promoter for 2-kb mRNA within exon 2 that represents nt 291–447 of 2.3-kb mRNA. However, the characterization of promoter elements that would drive the expression of the 2.3-kb mRNA have not, so far, been reported. Mullick et al. (24) have also provided evidence that suggests that the 2.0-kb mRNA, and not the 2.3-kb mRNA, is the predominant CYP27 mRNA in rat liver.

In the current report, we revalidate that the 2.3-kb mRNA is the predominant form of CYP27 mRNA in rat liver and thus disagree with the report of Mullick et al. (24). We present an analysis of the mode of CYP27 mRNA translation and how uORFs can regulate the initiation of its translation from the seventh AUG. Our results demonstrate that uORF1 regulates the synthesis of a 55-kDa CYP27 precursor in a way that prevents its accumulation in the cytosol. This regulatory mechanism, however, is lost with the deletion of uORF1, which also allows initiation of translation to take place equally from the sixth and the seventh AUGs. The presented evidence of uORF1 expression establishes the bicistronic nature of wild type 2.3-kb CYP27 mRNA. It is also suggested that following the uORF1 translation, synthesis of CYP27 protein involves reinitiation of translation from the seventh AUG. Together, these observations provide further proof that uORFs in mammalian mRNAs, like those in yeast, may manage access of ribosomes to authentic downstream AUG sites and regulate their translation accordingly (25, 26).

EXPERIMENTAL PROCEDURES

Materials—The chemicals and biochemicals used were of the highest purity grades purchased from Sigma or Fisher. Radiolabeled materials such as [3H]PepTcTP, [3H]PepPcTP (600 Ci/mmol) methionine (900 Ci/mmol) were purchased from Amersham Biosciences. Nytaran and nitrocellulose membranes for various blot transferswere obtained from Schleicher & Schuell. Alkaline phosphate-conjugated secondary antibody, avian myeloblastosis virus reverse transcriptase, RNasin, rabbit reticulocyte lysate (RRL), and some restriction enzymes were purchased from Promega. The AmpliTaq PCR fragment sequencing kit and PCR kits were supplied by PerKinElmer Life Sciences. The in vitro transcription kit and other restriction enzymes were purchased from Stratagene. Reagents for polycrylamide gel electrophoresis were purchased from Bio-Rad. The sources for pCMV4 vector and SPI-2 have been described before (15).

Bicistronic Plasmid Constructs—The wild-type 2.3-kb cDNA (WT2.3) cloned into Bluescript vector (WT2.3BS) and pCMV vector (WT2.3pCMV) has been described previously (15, 21). Also, WTd410 represents the 1.9-kb cDNA as described before (1). The deletion constructs WTd160 and WTd331 were prepared from WT2.3BS by using restriction sites EcoRV-XhoI and EcoRV-SmaI, respectively. Another deletion construct, WTd291–331 was prepared by using exonuclease III according to Sambrook et al. (27). Internal deletion constructs, WTd291–331 and WTd291–410, were prepared as follows. An antisense primer PrDel-1 (5’-GTAGTCCATTTGACCCTCTCAAGAACG-3’) with 5’ overhangs of 7 nt (underlined) complementary to nt 331–337 of WT2.3 was synthesized and used to amplify the terminal 290 nt of 5’-UTR (5’UTR1-290) with M13 reverse primer from the vector. Also, sense primer PrS-3 (5’-GGGATACACCTCGGCTTTTAA-3’) was used to amplify the downstream translated region 331–2300 with M13 forward primer from the vector. The two amplified fragments were mixed in an equimolar ratio, denatured at 95 °C for 4 min, and chilled on ice, and the fragments that annealed at their 5’-ends were first extended with T4 DNA polymerase and then amplified with T3 and T7 primers. The same procedures were employed to generate the deletion mutant WTd291–410 using PrDel-2 (5’-AGCCGGAATTGACCCTCTAACAAGACG-3’) and PrS-4 (5’-CTTGCGCTTCTAACTTCTTG-3’) primers. The amplified products with 291–331-nt and 291–410-nt deletions were cut with NotI and BamHI and then cloned into Bluescript vector (pBluescript II KS+) in the right orientation in order to form CW2.3 and CWd331. The WTd445 construct was generated by blunt ending a 1.5-kb CAT fragment at the 5’-end in the right orientation as described above (28). Similarly, three bicistronic constructs were prepared for translation from the seventh AUG. The amplified products with 291–331-nt and 291–410-nt deletions were cut with NotI and BamHI and then cloned into pCDNA3 vector for transfection in COS cells.

CAT Plasmid Constructs—A CAT construct WT-IFCAT was generated by cutting WTm448 with ClaI and then partially with SmaI to release the 1850-nt fragment. The 1850-nt fragment was replaced with a 1.5-kb Accl-HincII CAT fragment isolated from pCAT-Basic (Promega), which leaves CAT AUG in-frame from the sixth AUG of CYP27 Thr75. CAT plasmid construct CAT was generated by cloning the Clal site and then cloning a 1.5-kb Hincl-HincII CAT fragment isolated from pCAT-Basic instead of Accl and HincII CAT fragment. In this way, CAT AUG is inserted out-of-frame from the sixth AUG. CAT constructs d331-IFCAT and d333-OFcat were prepared by deleting the HindIII and SmaI fragment from the 5’-ends of WT-IFCAT and WT-OFCAT, respectively. A CAT construct, d280-IFCAT, was prepared by using exonuclease III. The CAT control was prepared by cloning HincII-cut 1.5-kb CAT fragment in pCMV4 (CATpCMV).

Bicistronic Plasmid Constructs—Three bicistronic plasmids, CW2.3, CW4.3, and CWd445, containing CAT as the first cistron and CYP27 as the second cistron with varying lengths of the CYP27 5’-UTR separating the two cistrons were constructed for in vitro expression and in vitro transcription and translation studies as follows. The restriction site deletion constructs were first isolated from pCAT-Basic instead of Accl and HincII CAT fragment. In this way, CAT AUG is inserted out-of-frame from the sixth AUG. CAT constructs d331-IFCAT and d333-OFcat were prepared by deleting the HindIII and SmaI fragment from the 5’-ends of WT-IFCAT and WT-OFCAT, respectively. A CAT construct, d280-IFCAT, was prepared by using exonuclease III. The CAT control was prepared by cloning HincII-cut 1.5-kb CAT fragment in pCMV4 (CATpCMV).

Epitope Tag Plasmid Construction—A DNA sequence encoding the c-Myc epitope, KLISEED, was inserted into the C-terminal end of the normal plasmid plasmid pCI1-CAT. The 3’ terminal end of the uORF1 of the CYP27 2.3-kb mRNA by manipulation within pCI1-CAT using two oligonucleotides (5’-GTCTGATCTCGCTGATCTGCT-3’ and 5’-AATGCTGACGAGGAGTCTGAG-3’) were synthesized with a 3’ overlap of 21 nt (underlined), representing the c-Myc epitope. PrEpi-1, an antisense primer, was used to amplify the 5’ region

FIG. 1. The unique characteristics of CYP27 mRNA 5’-UTR. A schematic representation is shown of the locations of sequence overlap with SPI-2 mRNA (nt 1–290), polyadenylation signal (nt 31–36), upstream in-frame AUGs (nt 173, 203, and 221), and out-of-frame AUGs (nt 288 and 294) with corresponding uORFs, upstream in-frame sixth AUG (nt 396) coding the 57-kDa extension of main ORF, the eighth AUG (nt 464) part of the main ORF, stop codons (nt 278 and 348), and stem loop structures (nt 183–203 and nt 356–375). For nucleotide sequence, see Ref. 15.
Translational Regulation of Cytochrome P-450c27

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In vitro transcription and translation, commercially available kits from Promega Biotech were used according to the manufacturer's instructions. Plasmid constructs WTm278, WTm450, WTm448, WTd331, and WT2.3 cloned in Bluescript vector were linearized with SpeI at the 3'-end of the coding sequence to produce "run-off" transcripts derived from the insert sequence using T3 RNA polymerase. RNA transcripts were quantified by the absorbance at 260 nm, and integrity was verified by gel analysis. Total RNA isolated from COS cells or RNA transcripts generated in vitro were used as templates for in vitro translation in an RRL system (33, 34). Each native RNA (10 μg) or RNA transcript (1 μg) was denatured at 65 °C for 5 min and then cooled to 37 °C in 20–30 min before being subjected to in vitro translation. In vitro translation products containing about 1 × 10⁶ acid-precipitable cpm were solubilized by boiling in 0.1 ml of a buffer containing 1% SDS. Twenty μl of solubilized translation product was directly loaded on the gel, whereas the rest was immunoprecipitated with polyclonal antibodies to CYP27 (20 μg of ammonium sulfate-fractionated IgG) or CAT essentially as described before (33, 34). The immunoprecipitated proteins were released by boiling in Laemmli's sample buffer, resolved by electrophoresis on a 12% polyacrylamide gel containing SDS (35), and gels were subjected to fluorography.

RESULTS

CYP27 mRNA Analysis—We used the RT-PCR technique to confirm our present (Fig. 2A) and previously reported (1, 15) Northern blot findings that 2.3-kb mRNA is the major mRNA species representing CYP27 in rat liver. Antisense primer PrAS-4 common to both mRNAs was used to synthesize complementary DNA strands from male and female CYP27 mRNAs by reverse transcription. This reverse transcription step was expected to generate 1.6-kb and 1.2-kb-long DNA strands in a ratio that would represent the levels of 2.3 and 2.0 kb mRNA in the liver, respectively. Since the 2.3-kb mRNA sequence is identical to the 2.0-kb mRNA sequence except for a 5’-UTR extension, it was possible to use a sense primer from the 5’-end of 2.3-kb mRNA (PrS-2) that would specifically amplify a 1.6-kb DNA strand by PCR, whereas the second sense primer used, PrS-5, would amplify both the 1.2- and 1.6-kb DNA strands. We used RNAs from COS cells transfected with WT2.3 and WTd410 to make sure that the two primer sets work equally well with the RT-PCR of both RNAs. The predictions that could be made as to the results of this experiment were as follows. First, if the 2.0-kb mRNA is the major species, the amplified product from PrS-5 and PrAS-4 should be severalfold higher than product formed between PrS-2 and PrAS-4. Second, if both mRNAs are expressed at the same level, then the product formed between PrS-5 and PrAS-4 should be at least 2-fold higher than product formed between PrS-2 and PrAS-4. Finally, if the 2.3-kb mRNA is the major species, then the amplified product from PrS-5 and PrAS-4 should be equal or slightly higher than the product formed between PrS-2 and PrAS-4.

The RT-PCR results shown in Fig. 2B convincingly demonstrate that 2.3-kb mRNA is the predominant species in rat liver. As expected, the levels of amplified product from PrS-5 + PrAS-4 and PrS-2 + PrAS-4 primer sets are almost identical, while the levels of amplified product from PrS-2 + PrAS-4 primer set are higher than those from PrS-5 + PrAS-4 primer set.
indicating that the amplified product from PrS-5 + PrAS-4 is mainly the result of 2.3-kb mRNA RT-PCR.

Mode of CYP27 mRNA Translation—In light of the unusual characteristics of CYP27 mRNA and its involvement in duplex formation, it was necessary to investigate how it undergoes translation. With its 5′-end blocked, the CYP27 mRNA could use IRES to undergo translation, whereas the two mRNAs are still in the hybrid form (36–39). This mechanism, if involved, would also explain why the seventh AUG and not the sixth AUG is used for initiation of translation. This mode of translation was investigated by preparing bicistronic constructs with CAT as the first cistron and CYP27 translated regions as the second cistron. The two cistrons were separated either by intact 5′-UTR (CW2.3) or partial 5′-UTR (CWD331) or none (CWD445) as shown in Fig. 3A. The three bicistronic clones, CW2.3, CW331, and CW445, along with WT2.3 were subjected to in vitro transcription. The run-off transcripts were translated in vitro in the RRL system, and the translation products were subjected to electrophoresis either directly or after immunoprecipitation with CYP27 or CAT antiserum on a polyacrylamide gel. If IRES is present immediately upstream to the seventh AUG, the CW2.3 and CW331 transcripts should express both CAT (first cistron) and CYP27 (second cistron) proteins. If IRES is present upstream to 331 nt, then only CW2.3 should express both cistrons. However, under identical conditions, CWD445 should only express CAT because no part of the 5′-UTR is separating the two cistrons. Fluorography results are presented in Fig. 3B. From the left, first three lanes show total in vitro translation products of CW2.3, CW331, and CW445 transcripts, which were resolved by electrophoresis. The CAT antibody was used as control. On the other hand, CAT antibody was able to detect CAT antigens in CW2.3-, CWD331-, and CWD445-transfected COS cells, while reacting to a 52-kDa protein in WT2.3-transfected COS cells used as control. The membranes were separately probed with monoclonal antibody to CYP27 and polyclonal antibody to CAT. Lanes marked Mock represent extracts from COS cells transfected with vector alone.

CYP27 and CAT as probes. The results, as shown in Fig. 3C, were identical to those obtained with cell-free extracts. The CYP27 antibody was unable to detect any CYP27 antigens in CW2.3-, CWD331-, and CWD445-transfected COS cells, while reacting to a 52-kDa protein in WT2.3-transfected COS cells used as control. On the other hand, CAT antibody was able to detect CAT antigens in CW2.3-, CWD331-, and CWD445-transfected COS cells and unable to identify one in WT2.3-transfected COS cells as expected. Thus, these results rule out the possibility that IRES is used as a mode of CYP27 mRNA translation.
The second possible mode of translation for a 5′-end-blocked CYP27 mRNA could involve digestion of the duplex by double strand-specific RNase, resulting in the conversion of the 2.3-kb mRNA into an mRNA of ~2.0 kb. This truncated mRNA will have sufficient 5′-UTR left for ribosomes to bind and initiate translation. Previously reported transient expression of 1.9- and 2.3-kb cDNAs for CYP27 in COS cells suggested that the translation is initiated from the same AUG that is the first in-frame AUG for 1.9-kb cDNA and the seventh in-frame AUG for the 2.3-kb cDNA (15). The 55-kDa precursor form thus results therefore suggest that 160–280-nt region in the 5′-UTR is needed to direct the initiation of translation exclusively from the seventh AUG. Interestingly, when the 5′ UTR1–290 region was ligated at the 5′-ends of WTd331 and WTd410 to form two internal deletion mutants (WTd291–331 and WTd219–410, respectively), they behaved like WT2.3 in COS cells, since only 52-kDa CYP27 was identified on the Western blot (Fig. 4B). These results, while supporting the concept that for the initiation of translation to take place exclusively from the seventh AUG, the 160–280 nt region of the 5′ UTR1–290 is needed, they rule out the possibility of dsRNase being involved in the processing of duplex RNA. This was further substantiated by failing to identify a truncated mRNA in the COS cells cotransfected with WT2.3 and SPI in a 1:4 ratio as described before (21). As shown in Fig. 5, the RNA from cotransfected COS cells was subjected to S1 nuclease protection using a 289-nt single-stranded probe (nt 159–448) as described before (15). The S1 protected fragments were melted and fractionated through a 5% acrylamide, 8.3 M urea sequencing gel.

![Translation Regulation of Cytochrome P-450c27](image-url)
sent the truncated mRNA. We do see a lesser amount of protected fragment in lane 1 compared with lane 2, which is most likely due to competition between SPI mRNA and the S1 probe (WTm448) do not prevent expression of CYP27 protein, as is the case of d331-IFCAT and d280-IFCAT, in the second making the context around the seventh AUG (46, 47). We prepared two mutant transcripts that in cotransfected COS cells determined by RNase protection assay as reported under “Experimental Procedures.” The protected RNAs were analyzed on a 6% acrylamide, 8 M urea gel. The RNA from pCMV vector-transfected COS cells was used as a negative control, and in vitro synthesized CAT RNA (471-nt probe) was used directly as the positive control. CP, chloramphenicol; ACP, acetyl-C.

Regulation of CYP27 mRNA Translation from the Seventh AUG—The regulatory role of the 5′-UTR in the CYP27 mRNA translation from the seventh AUG was investigated by replacing the CYP27 translated region (1850 nt) with CAT as a reporter gene. The CAT was placed both in-frame (WT-IFCAT) and out-of-frame (WT-OFCAT) to the sixth AUG. We also generated two deletion mutants, d331-IFCAT and d280-IFCAT, from WT-IFCAT and one deletion mutant, d331-OFCAT, from WT-OFCAT as depicted in Fig. 6A. These constructs were expressed in COS cells, and after making sure that the transcript levels for all constructs were almost identical (Fig. 6D), CAT activity in the COS cell extracts was analyzed and compared with CAT control (CATpCMV). As shown in Fig. 6, B and C, both WT-IFCAT and WT-OFCAT, when expressed in COS cells, resulted in CAT activity that was reduced by 41–52% compared with control CATpCMV. However, when their deletion mutants d331-IFCAT, d280-IFCAT, and d331-OFCAT, were expressed in COS cells, opposing effects on CAT activity were observed. Whereas deletion of 280 nt (d280-IFCAT) or 331 nt (d331-IFCAT) from the WT-IFCAT increased the expression from 52 to 63% or 82% of the control, respectively, the deletion of 331 nt (d331-OFCAT) from WT-OFCAT reduced expression from 42 to 10%. This profound reduction in the CAT activity when d331-OFCAT was expressed in COS cells is most likely due to shift in the initiation of translation to the sixth AUG, which in this case being out-of-frame from the CAT initiation codon resulted in further reduction in the CAT expression. Whereas in the case of d331-IFCAT and d280-IFCAT, the shift in the initiation of translation to the sixth AUG will still lead to the CAT expression; hence, no reduction in the rate of inhibition is observed. These results exactly mimic our Western blot results by showing that uORF1 is regulating the translation from the seventh AUG and, in its absence, the initiation of translation is partially shifted to the sixth AUG.

The results presented in Fig. 6D also rule out the possibility that reduction in the accumulation of CYP27 antigen in the presence of an intact 5′-UTR could be due to suppression of 2.3-kb mRNA synthesis by the presence of a polyadenylation signal at the 5′-proximal end of CYP27 mRNA. Almost identical levels of CAT transcripts observed in Fig. 6D indicate that the polyadenylation signal present in the 5′-terminal region of WT-OF CAT and WT-IFCAT did not suppress their mRNA synthesis and that the observed reduction in the CAT activity was due to the regulatory role of 5′UTR1–290. In other words, the polyadenylation signal in the 5′-UTR is silent and, most likely, bears no influence on the regulation of CYP27.

Alterations in Nucleotide Context of the Seventh AUG and Its Effect on the Initiation of Translation—In CYP27 mRNA, the seventh AUG with CGATCTAUGG nucleotide context is used for the initiation of translation in liver as well as in transfected COS cells (15). However, under certain conditions, as described above, the sixth in-frame AUG with GCCTGGAUGG nucleotide context is also used for initiation of translation. Comparing these two nucleotide contexts with the optimal context (GCC(A/G)CCAUGG), it appears that both the sixth AUG and the seventh AUG are moderately strong. However, with the absence of a purine at −3-position in both of these AUGs, the presence of GCC at −6 to −4 in the sixth AUG context should have made it more preferable for initiation of translation over the seventh AUG (46, 47). We prepared two mutant transcripts from the WT2.3, in one completely eliminating the seventh AUG (WTm448) and in the second making the context around the AUG codon weaker by replacing G by C (WTm450). As shown in Fig. 7B, both the weaker context around the seventh AUG (WTm450) and elimination of the seventh AUG (WTm448) do not prevent expression of CYP27 protein, as is

Fig. 6. Role of 5′-UTR in the regulation of CYP27 mRNA translation from the seventh AUG. A, a schematic representation of various CAT constructs that were used for transfection experiments. Open boxes represent CAT gene, and arrows show the translational initiation sites. C, the CAT plasmids that contain different lengths of CYP27 5′-UTR between the SV40 promoter and CAT gene in in-frame and out-of-frame orientations to the sixth AUG were transfected in COS cells for the CAT assay. Cytosolic fractions were prepared from transfected COS cells and subjected to in vitro reconstitution of CAT activity. The CAT activity was quantified by a PhosphorImager and autoradiographed on an x-ray film. B, relative CAT activity values that are averages ± S.D. of three independent experiments, calculated with CAT activity of CATpCMV as 100%. D, the levels of CAT reporter RNA in transfected COS cells determined by RNase protection assay as reported under “Experimental Procedures.”
evident from the accumulation of 52-kDa mature form in COS cell mitochondria. However, reduction in the accumulation of CYP27 antigen by almost 50% is clearly evident. The possibility that the initiation of translation in the case of WTm448 might have shifted to the sixth AUG was investigated by subjecting RNA isolated from WTm448- and WTm450-transfected COS cells to in vitro translation and immunoprecipitation. The precursor forms identified were compared with that of WT2.3 and WTd331, which were previously shown to synthesize 55-kDa precursor forms, respectively. As shown in Fig. 7C, a 55-kDa precursor form was identified with WTm450, suggesting that the initiation of translation continues to take place from the much weaker seventh AUG, whereas with WTm448 COS RNA we identified a 54.5-kDa precursor form on the gel, suggesting that elimination of the seventh AUG shifted the initiation of translation to the eighth in-frame weak AUG codon (AGCCGCAUGA). Since the eighth AUG is situated 15 nt downstream of the seventh AUG, we see an immunoprecipitated band moving slightly faster than the 55-kDa precursor. This eighth AUG normally forms a part of mature translation product. However, we have previously shown that an incomplete 1.85-kb cDNA (eighth AUG as its first AUG) of CYP27 was able to weakly express when transfected in COS cells (1). These results suggest that the regulatory elements present upstream of the sixth AUG (most likely within the 5’UTR—290) are helping scanning ribosomes to bypass the sixth AUG and initiate translation exclusively from the seventh AUG.

**Role of uORF1 in Directing Initiation of Translation from the Seventh AUG**—The most prominent regulatory elements present in the nt 160–280 region of 5’-UTR that is needed for the correct initiation of translation are three fully overlapping uORFs of 105 (uORF1), 72 (uORF2), and 57 (uORF3) nt that are terminated by the same stop codon, which is in-frame with the seventh AUG. The AUG codons for uORF1 and uORF3 have a moderately strong context, whereas uORF2 has the strong AUG context. However, the presence of a secondary stem loop structure just downstream of first AUG should enhance the fidelity of initiation from this AUG (48). In order to determine the role of these overlapping uORFs in CYP27 regulation, we eliminated the stop codon by site-directed mutagenesis, which resulted in a 2123 nt-long open reading frame that should be capable of translating CYP27 protein. As shown in Fig. 8D, the expression of mutated construct WTm278 in COS cells resulted in the accumulation of two CYP27 antigen bands of 52 and 67 kDa on the Western blot, suggesting that in the absence of stop codon the initiation of translation has shifted most likely from the seventh to the first AUG. The mature 52-kDa protein identified could either be the result of mitochondrial processing of the 67-kDa precursor or of translation simultaneously taking place from the seventh AUG, resulting in the accumulation of mature form as normally observed with WT2.3. To determine whether initiation of translation is exclusively shifted to the first AUG or carried out from both the first and the seventh AUGs, RNA from the WTm278-transfected COS cells was subjected to in vitro translation and immunoprecipitation. The results shown in Fig. 8C indicate that the initiation of translation is exclusively taking place from the first AUG and that the mature form is the result of mitochondrial processing of 67-kDa precursor. These results suggest that CYP27 mRNA might be a bicistronic mRNA under normal conditions that in addition to CYP27 protein is also translating uORF1 into a 35-amino acid-long polypeptide, which was confirmed in the following results.

**Preparation and Expression of Epitope-tagged uORF1 Confirms Bicistronic Nature of CYP27 mRNA**—The bicistronic na-
nature of CYP27 mRNA was investigated by inserting a c-Myc epitope (KLISEED) prior to the C-terminal end of the uORF1 (WTEpi). Placing the epitope at the C terminus of the uORF1 would also permit the detection of uORF2, if translation was initiated from the second AUG, which is by far the strongest AUG codon in the CYP27 5′-UTR. The insertion of the epitope in-frame and in the right orientation was confirmed by sequencing. The expression of uORF1 in COS cells was expected to result in the synthesis of a protein of about 5.5 kDa that should be immunoprecipitated with monoclonal antibody to c-Myc. WTEpi was transfected in COS cells, and 44 h post-transfection, cells were pulse-labeled with [35S]Met at 200 μCi/ml for 2 h. The cell lysates were immunoprecipitated with c-Myc epitope-specific primary antibody (1 μg) resolved by electrophoresis on 12–20% gradient SDS-polyacrylamide gel and subjected to fluorography.

**DISCUSSION**

The regulation of cytochrome P450 enzymes both at the transcriptional and posttranscriptional levels has been reported in the eukaryotes. Whereas the molecular mechanisms of induction, such as by the mediation of receptors, transcriptional regulation, and stabilization of mRNA or of enzyme proteins, is well understood, the repression of P450s synthesis is far less understood. The fact that the encumbered 5′ noncoding sequences characteristic of genes for regulatory proteins such as growth factors, cytokine receptors, protooncogenes, etc. are often involved in the extensive regulation of the regulators at the level of translation and/or RNA processing suggest the similar involvement of the encumbered 5′ noncoding sequence of CYP27 mRNA in its posttranscriptional regulation (22, 23).

The repression of CYP27 mRNA translation by the formation of an RNA duplex between the complementary sequences of CYP27 and SPI mRNAs was previously demonstrated in our laboratory (21). The repression of translation was accredited to 5′-end blocking of CYP27 mRNA and subsequent prevention of the 40 S ribosomal docking at the 5′-end, since the scanning hypothesis of eukaryotic protein synthesis postulates that the initial contact between protein components (initiation factors and 40 S ribosomal unit) and mRNA occurs at the 5′-end of the polypeptide chain (22, 23). Cotransfection of SPI and CYP27 cDNAs at a 1:1 ratio was shown to significantly suppress the translation of CYP27 mRNA (by about 50%) in COS cells. However, an increase in the ratio of SPI/CYP27 cDNAs to 4:1 enhanced inhibition only up to 66% compared with about 90% involvement of CYP27 mRNA in duplex form (21). These results suggested that duplex must be undergoing processing to release CYP27 mRNA for translation. With the reestablishment of the predominance of the 2.3-kb mRNA in rat liver by validating our very convincing Northern blots results (Fig. 2A) by RT-PCR (Fig. 2B), it became necessary to establish the mode of CYP27 mRNA translation subsequent to duplex formation. The available cellular mechanisms suggested three possible ways for 5′-end blocked CYP27 mRNA (Fig. 1) to undergo translation. First, translation takes place via involvement of a cap-independent IRES, whereas two mRNA are still in the duplex form (36–39). Second, the dsRNase digests the duplex and releases a truncated mRNA with enough 5′-UTR left for ribosomes to bind and initiate translation (49–51). Finally, translation occurs following the separation of two strands of the duplex either by the action of RNA helicases or by the dsRNA unwindase activities present in the liver (40–43).

In principle, both viral and cellular mRNAs containing unusually long leader sequences with multiple short upstream open reading frames are good candidates for translation initiating via a cap-independent IRES (52–57). This mechanism does not require a 5′ cap structure or scanning through the greater part of the 5′-UTR and the bicistronic assay is considered the most valid test to examine internal initiation (58). The presence of IRES in the CYP27 5′-UTR was investigated by preparing bicistronic constructs with CAT as the first cistron and CYP27 as the second cistron. The two cistrons were separated either by whole 5′-UTR (CW2.3) or partial 5′-UTR (CWD331) or directly ligated without any 5′-UTR (CWD445) separating the two cistrons. It was predicted that if IRES is present in the 5′-UTR, the CWD445 should be unable to express the second cistron, while depending upon the position of the IRES the CW2.3, and the CWD331 should be able to express both cistrons. However, with all of the three-bicistronic constructs, the second cistron was unable to express both in vivo and in vitro, suggesting that CYP27 mRNA does not use IRES. Thus, these results ruled out the involvement of IRES as a mode of CYP27 mRNA translation.

The presence of dsRNases in eukaryotic cells and their role in the digestion of double-stranded RNAs is well established (49–51). The duplex if digested by dsRNase would result in a truncated mRNA of ~2.0 kb. We investigated the expression of this truncated mRNA in COS cells by preparing several 5′ progressive and internal deletion mutants from WT2.3 as shown (Fig. 4A). In view of the fact that initiation of translation from both the sixth and the seventh AUGs can lead to the synthesis of CYP27 precursors, it was previously established in liver (15) and here again in COS cells (Fig. 7C) that 2.3-kb CYP27 mRNA translation is initiated exclusively from the sev-
hadnt AUG to synthesize a 55-kDa precursor in vivo, which after transport to mitochondria is truncated to a 52-kDa mature protein (1, 15, 21). However, when WTd280 (representing possible dsRNase-truncated 2.0-kb mRNA) and WTd331 (closely related to the 2.0-kb mRNA sequence reported by Mullick et al. (24)) were expressed in COS cells, two precursor forms of 55 and 57 kDa were identified, indicating the initiation of translation from both the sixth AUG and the seventh AUG. In comparison, only the 52-kDa band is identified in WT2.3 control initiation of translation from the seventh AUG. Consequently, the requirement for intact 5’-UTR to correctly initiate CYP27 mRNA translation suggests that release of intact CYP27 mRNA from the duplex is most likely achieved either by the action of RNA helicases that unwind double-stranded RNAs by virtue of binding to single-stranded regions of the RNAs (40) or by the action of a novel double strand-specific dsRNA unwindase activity that has been shown to efficiently and permanently separate two strands (<100 base pairs) of RNA duplex (44, 45). Although, dsRNA unwindase activity mainly resides in the nucleus, its presence and activity in the cytoplasm has been confirmed (44, 45). Furthermore, the possibility that RNA helicase and/or dsRNA unwindase activities and not dsRNase are involved in the processing of the duplex was substantiated by the failure to identify a truncated 2-kb RNA in the COS cells cotransfected with CYP27 and SPI cDNAs (Fig. 5).

The cap-dependent translation of 2.3-kb CYP27 mRNA suggests extensive regulation at the level of translation, since the presence of uORFs are known to interfere with ribosome scanning and initiation of translation at the authentic AUG start site (40). Systemic mutagenesis of nucleotides in the vicinity of the AUG codon has revealed that GCC/A/GCCAUGG is the optimal context for initiation of translation in cultured cells (48, 61–65). In particular, G at +4 (with A of AUG as +1) and a purine (A or G) at −3 have the strongest effect in the selection of initiation codon. The effects from the other nucleotides such as GCC at −6 to −4-positions are observed mostly in the absence of −3 purine or G+4. In CYP27 mRNA, both the sixth (GCCCTGAUGG) and the seventh (CGATCTAUGG) AUGs can be considered moderately strong, since both have a G at position +4 and are missing a purine at −3 position. However, with GCC at −6 to −4-positions and preceding the seventh AUG, the sixth AUG looks to be a better choice for the scanning ribosomes to initiate translation. However, knowing that control over initiation of translation from the seventh AUG resides within nt 160–291 of the 5’-UTR, we wanted to know whether that control is imparted through the expression of uORF1.

Reports in eukaryotes of full-length proteins being translated from a single cellular bicistronic mRNA are rare (66–68), but it has been demonstrated that such transcripts can arise from differential exon splicing (68). A single mammalian transcript with tandem reading frames encoding two proteins have been described (66, 69). Whereas deletion of the terminal 280 nt (WTd280) from CYP27 mRNA results in the synthesis of two full-size proteins initiated from the sixth and the seventh AUGs, elimination of the stop codon that terminates uORF1 results in the expression of a single 67-kDa protein by completely shifting the initiation of translation from the seventh AUG. This suggests that the ribosome preinitiation complex does recognize the first AUG, and, therefore, it is very likely that uORF1 is expressed under normal conditions. Subsequently, expression of uORF1 in COS cells was confirmed by labeling it with c-Myc epitope and identifying a 5.5-kDa amino...
acid peptide by pulse labeling and immunoprecipitation analyses (Fig. 9). By establishing that the expression of the uORF1 is most likely responsible for regulating the CYP27 mRNA translation from the seventh AUG. However, what makes the scanning ribosomes bypass the sixth AUG that could also result in the synthesis of CYP27 was not clear.

Multiple features of an mRNA have been shown to influence the efficiency with which ribosomes reinitiate downstream translation after translation of a uORF. The most prominent is the short intercistronic region that can prevent reinitiation from the immediate downstream AUG (70, 71). However, studies with several systems reveal that the sequence of the intercistronic region, not just its length, can affect reinitiation (72–74). Introduction of hairpin structure downstream to the uORF may promote ribosome release, leading to destabilization of the mRNA (75). In addition to the intercistronic region, sequences upstream to and within the uORF and even the particular downstream cistron all can affect reinitiation efficiency, demonstrating that the control of reinitiation is very complex (62, 72, 76). The 115-nt intercistronic region between stop codon that terminates uORF1 and the sixth AUG does contain overlapping out-of-frame uORF4 and uORF5 and in particular a hairpin structure just upstream to the sixth AUG that might be promoting the release of scanning ribosomes. Thus, the 40 S ribosomal subunit may not have had sufficient time to reacquire Met-tRNA to reinitiate translation from the sixth AUG, and instead subsequent translation must occur by reinitiation at the next in-frame AUG that, in this case, is the seventh AUG. The results from other systems document that some downstream AUG codons are skipped when ribosomes scan in the reinitiation mode (72, 73). Furthermore, since translation occurs via linear scanning, the deletion of the seventh AUG shifts initiation of translation to the eighth in-frame AUG with even weaker context (GCCGCAUGA), which otherwise forms a part of the translated region. Thus, these results suggest that after termination of uORF1 translation, the scanning ribosomes are unable to reinitiate at the sixth AUG, most likely due to sequence characteristics of the 115-nt intercistronic region, and reassemble near the seventh AUG to initiate translation. However, when the uORF1 is deleted (WT280), the same intercistronic region, which now becomes the 5′-terminal region for WT280, somehow loses those sequence characteristics that prevent reinitiation from the sixth AUG and allows initiation of translation from the sixth AUG and reinitiation at the seventh AUG. In the well studied case of the Saccharomyces cerevisiae GCN4 gene, uORFs regulate protein synthesis by affecting which downstream AUG codons are utilized by reinitiating ribosomes (26). Ribosomes translate the first uORF in the GCN4 mRNA under all conditions. They then reinitiate at another uORF when amino acids are plentiful or, under starvation conditions, they bypass the other uORFs and reinitiate at the GCN4 start codon. Several other uORFs have been shown to act by a mechanism that depends on the uORF-encoded peptide sequence and, in some cases, involves ribosomal stalling on the mRNA (26, 67, 72, 77–82). For the vast majority of uORFs, insufficient data are available to enable predictions about whether they affect downstream translation and, if so, about the mechanism involved.

In summary, we have described a translational regulatory mechanism for CYP27 that mediates the repression of its mRNA translation and in the process identified a new post-transcriptional regulatory process for a gene from the cytochrome P450 family. The need for an extensive regulation of CYP27 mRNA translation could be explained by its involvement in the rate-limiting step in the catabolic conversion of cholesterol to bile acids and in the activation of vitamin D3 to 1,25-dihydroxy D3 which not only plays a critical role in the activation of genes that control intestinal calcium transport, but also controls the expression of many genes involved in a plethora of biological actions. Many of these nonclassic responses have suggested a number of therapeutic applications for 1,25-dihydroxy D3 and its analogs (13, 83).

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