Two Promoters in the Bovine Adrenodoxin Gene and the Role of Associated, Unique cAMP-Responsive Sequences†

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Received September 17, 1991; Revised Manuscript Received November 25, 1991

ABSTRACT: The bovine adrenodoxin gene gives rise to two species of mRNA differing only at their 5′-ends. The synthesis of these two types of mRNA in bovine adrenal cortical cells is regulated transcriptionally in part by ACTH via the action of cAMP. Examination of the 5′-end of the adrenodoxin gene revealed that each mRNA contains sequences derived from a different exon encoding the mitochondrial leader sequence. To define the sequences necessary for the synthesis of these two types of mRNA and to determine if the synthesis of each is driven by a separate promoter, 5′-regions of the adrenodoxin gene were inserted upstream from two different reporter genes and tested for promoter/enhancer regulatory activity by transient transfection into mouse adrenocortical Y1 tumor cells. The results clearly demonstrated that the bovine adrenodoxin gene contains two functional promoters; one, ADXP1, located in the 5′-flanking region gives rise to the minor form of mRNA, and a second, stronger promoter, ADXP2, which maps within intron 1 gives rise to the major form of mRNA. Unique cAMP-responsive sequences were found upstream from each promoter which share no sequence homology to the consensus CRE (*c*AMP-*r*esponsive *e*lement). Upon transient expression, the cAMP-responsive sequence associated with the ADXP2 promoter, termed CRS2, confers the cAMP responsiveness to stimulate the transcription of the linked β-globin reporter gene regardless of whether the adrenodoxin ADXP2 promoter or the β-globin promoter was utilized. As to the cAMP-responsive sequence located in the 5′-flanking region upstream to the ADXP1 promoter, which is termed CRS1, cAMP responsiveness was observed only with the heterologous β-globin promoter and not with the ADXP1 promoter. Thus, it is not clear whether CRS1 functions in vivo as a cAMP-responsive element. The cAMP-stimulated expression is not abolished by protein synthesis inhibitors, suggesting that newly synthesized protein factors are not required for cAMP-regulated expression of the linked reporter genes. Both adrenodoxin promoters function in hepatocyte (HepG2) and kidney (COS 1) cell lines derived from nonsteroidogenic tissues which are known to express adrenodoxin.

The iron–sulfur protein adrenodoxin is a key electron-transport component of mitochondrial hydroxylation systems. From NADPH, electrons are shuttled through a flavoprotein, adrenodoxin reductase, to adrenodoxin and subsequently to a mitochondrial P-450 monooxygenase. The specificity of the mitochondrial hydroxylation reactions resides in the P450 enzymes, different forms of which are specific for cholesterol side chain cleavage or 11β-hydroxylation in the pathways of steroid hormone biosynthesis [reviewed by Waterman and Simpson (1989)], sterol 26-hydroxylation in the bile acid biosynthetic pathway (Pederson et al., 1977; Anderson et al., 1989), and 1α-hydroxylation of 25-hydroxyvitamin D in the vitamin D activation pathway (Driscoll & Omdahl, 1986). At present, it can be postulated that all mitochondrial P450 systems, including those reported to metabolize xenobiotic compounds (Raza & Avadhani, 1988), will utilize adrenodoxin reductase and adrenodoxin to capture reducing equivalents from NADPH. Adrenodoxin is relatively abundant in steroidogenic tissues, and present to a lesser degree in liver, kidney, and brain (Le Goascogne et al., 1987). It is likely that adrenodoxin will be found in other tissues as well since it can be expected that additional forms of mitochondrial cytochrome P450 have yet to be discovered.

Like most mitochondrial proteins, adrenodoxin is encoded by a nuclear gene as a higher molecular weight precursor which is processed into the mature form upon import into mitochondria (Matocha & Waterman, 1984, 1985). Characterization of transcripts derived from the bovine adrenodoxin gene has revealed the presence of two distinct mRNA species that differ only at their 5′-terminal sequences (Okamura et al., 1987). Each mRNA type encodes the same mature adrenodoxin protein, the differences residing in the leader peptide sequences. Examination of the 5′-end of the bovine adrenodoxin gene has shown that exon 1 encodes one leader peptide sequence, exon 2 encodes the other, and exons 3, 4, and 5 encode the amino acids of the mature adrenodoxin (Kagimoto et al., 1988; Sagara et al., 1990). The leader peptide sequence encoded by exon 1 contains an in-frame stop codon, and consequently transcripts containing this sequence cannot be translated into a functional iron–sulfur protein (Kagimoto et al., 1988; Sagara et al., 1990). The predominant form of adrenodoxin mRNA which accounts for 90% of the total adrenodoxin mRNA isolated from bovine adrenocortical cells (Okamura et al., 1988) starts from exon 2 while the less abundant mRNA starts from the first exon (Kagimoto et al., 1988). The sequence from exon 2 is then presumably removed from this minor mRNA species through the process of alternative splicing. The levels of both types of adrenodoxin

† This work was supported in part by American Heart Association, Texas Affiliate, Grant-in-Aid Award 90G-096 (J.-Y.C.), USPHS Grant DK 28350 (M.R.W.), and Robert A. Welch Foundation Grant I-624 (M.R.W.).

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0006-2960/92/0431-2400$03.00/0

mRNA in the adrenal cortex are enhanced by the peptide hormone adrenocorticotropin (ACTH)[1] through the action of intracellular cAMP (Okamura et al., 1988), apparently at the transcriptional level (John et al., 1986).

In order to define the sequences associated with the bovine adrenodoxin gene which are necessary for transcription of these two mRNA species and to determine if each is driven by a separate promoter, we have characterized the promoter/enhancer sequences within this gene, with the aim of understanding their functions and of eventually identifying nuclear factors that they may bind. Recombinant plasmids containing different portions of the bovine adrenodoxin gene linked to different reporter genes were generated and transfected into the mouse adrenocortical Y1 tumor cell line, and the effect of adrenodoxin DNA on the transient expression of the reporter genes was measured. Our results clearly demonstrate that the bovine adrenodoxin gene contains two separate, functional promoters and that upstream from each is present a unique cAMP-responsive element.

Experimental Procedures

Construction of Recombinant Plasmids. To characterize the promoter/enhancer regulatory sequences in the bovine adrenodoxin gene, two reporter genes were used to construct chimeric plasmids for transient expression experiments, the bacterial chloramphenicol acetyltransferase gene (CAT) and the rabbit β-globin gene (OVEC). As will be seen, the pADX-CAT, pADXP1-OV, pADXP2-OV, and pADXP1P2-OV constructs utilize the homologous adrenodoxin promoters for initiation of transcription while the pADXF-OV and pADXI-OV constructs utilize a minimal rabbit β-globin promoter for this purpose. Construction of plasmids pOV-1, pSV-OV, pOV-Ref, and pCRE(3X)-OV has been described previously (Westin et al., 1987; Lund et al., 1990).

Construction of pADX-CAT Fusion Genes. Three DNA fragments spanning different regions of the 5′-terminus of the bovine adrenodoxin gene were cloned into the promoterless CAT plasmid pBS-CAT (Figure 1A). Plasmid pBS-CAT was obtained by subcloning the *Bam*HI fragment containing the CAT structural gene from mCAT (Kruse et al., 1988) into Bluescript (Stratagene). Adrenodoxin sequences ranging from −2 kb to +808 bp, from −675 to +72 bp, and from +249 to +808 bp were inserted into pBS-CAT at the position next to the 5′-boundary of the CAT gene to yield pADX2.8K-CAT, pADX1-CAT, and pADX2-CAT, respectively.

Construction of pADXP1-OV, pADXP2-OV, and pADXP1P2-OV Fusion Genes. Plasmid pOV-1 is derived from pUC18 and contains the rabbit β-globin minimal promoter and its coding sequence (Westin et al., 1987) and was kindly made available to this laboratory by Dr. Walter Schaffner of the University of Zurich, Switzerland. The promoterless vector pOV-3 was constructed by restricting pOV-1 with *Sal*I and *Pst*I to remove the β-globin promoter sequence. The linearized pOV-1 was ligated with a piece of multiple cloning sequence obtained from Bluescript by *Xho*I and *Pst*I restriction digestion. Bovine adrenodoxin sequences ranging from −675 to +72 bp and from +249 to +808 bp were subsequently inserted into pOV-3 at the position 13 nucleotides upstream from the β-globin structural gene to yield pADXP1-OV and pADXP2-OV, respectively (Figure 1B). Plasmid

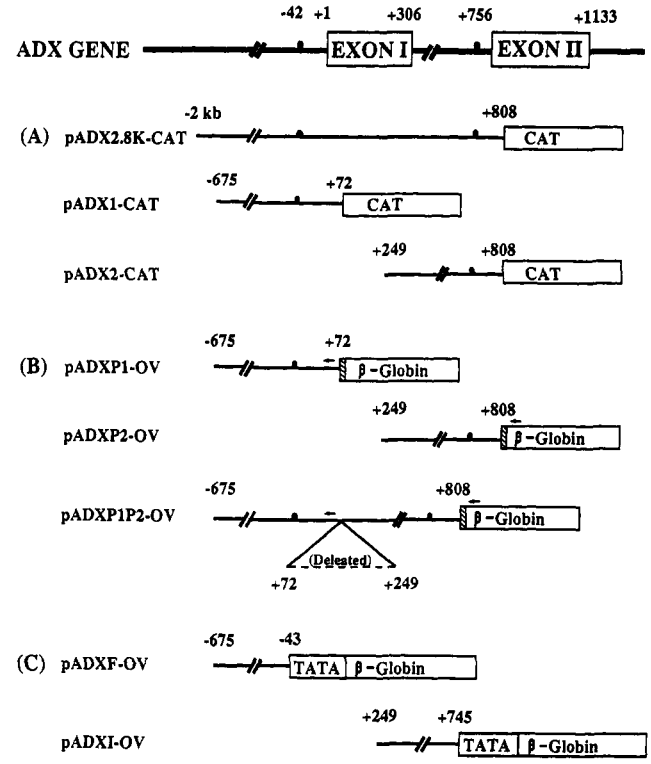

FIGURE 1: Schematic representation of recombinant pADX-CAT and pADX-OV plasmids. The structure of the 5′-end of the bovine adrenodoxin gene (ADX GENE) has been previously described, and the putative TATA boxes are indicated by closed circles (Kagimoto et al., 1988). Plasmids pADX-CAT and pADX-OV were constructed by linking different regions of the 5′-terminus of the bovine adrenodoxin gene to (A) the bacterial chloramphenicol acetyltransferase gene, (B) the rabbit β-globin structural gene, and (C) the β-globin minimal promoter and its structural gene as described under Experimental Procedures. The open boxes designated as CAT and β-Globin represent the sequences of CAT and β-globin structural genes, respectively. The hatched boxes in (B) represent the 5′-flanking sequence (−13 to −1 bp) derived from the β-globin gene. The boxes designated as TATA in (C) represent the minimal β-globin promoter sequence. The arrows shown in (B) indicate the positions where the specific primers used for primer extension analyses bind.

pADXP1P2-OV was constructed by inserting into pADXP2-OV the 0.75-kb adrenodoxin fragment obtained from restriction digestion of pADXP1-OV by *Sac*I and *Cla*I. Therefore, pADXP1P2-OV contains adrenodoxin sequences from −675 to +808 bp except for the region from +72 to +249 bp.

Construction of pADXF-OV and pADXI-OV Fusion Genes. Two adrenodoxin fragments ranging from −675 to −43 bp and from +249 to +745 bp were generated, respectively, by polymerase chain reaction (PCR) using Taq polymerase. A *Sal*I site was created at the 3′-end of the PCR fragments by using a 3′-primer containing an eight-base overhang (5′-GCGTCGAC-3′) at its 5′-terminus for the amplification reactions. The PCR-generated adrenodoxin fragments were then cloned into pUC19 at the *Sma*I site and completely sequenced to establish the absence of PCR errors. The adrenodoxin sequences (which are flanked by *Sal*I sites in the resulting pUC plasmids) were subsequently isolated and subcloned into pOV-1 at the *Sal*I site, producing pADXF-OV and pADXI-OV (F indicating the 5′-flanking sequence and I indicating the intron 1 region) (Figure 1C).

Cell Culture and Transient DNA Transfection. Mouse adrenocortical Y1 tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Forskolin treatment of these cells as de-

[1] Abbreviations: ACTH, adrenocorticotropin; SDS, sodium dodecyl sulfate; $P450_{17\alpha}$, 17α-hydroxylase cytochrome P450; $P450_{scc}$, cholesterol side chain cleavage cytochrome P450; $P450_{c21}$, steroid 21-hydroxylase cytochrome P450; bp, base pair(s); kb, kilobase(s).

scribed below was shown to increase the level of endogenous adrenodoxin 3–5-fold by immunoblot analysis using antibodies produced in this laboratory (Kramer et al., 1982) (data not shown). Cells were transfected by the calcium phosphate precipitation method according to Graham and van der Eb (1973). A plasmid carrying the guanine phosphoribosyltransferase (GPT) gene linked to the Rous sarcoma viral (RSV) promoter was cotransfected with the pADX-CAT constructs to provide an internal control to evaluate differences in transfection efficiency between different culture dishes. In general, 10 μg of pADX-CAT/with 0.5 μg of pRSV-GPT was used for transfection of a 100-mm dish 16–20 h after the cells had been passaged. The cells were incubated with the $Ca_3(PO_4)_2$–DNA precipitates for 4 h followed by glycerol shock for 3 min. The cells were then cultured in fresh medium and incubated for an additional 16 h prior to the addition of forskolin (25 μM final concentration). After 24 h, the cells were harvested, and CAT and GPT activities were measured in the cell lysates as described (Fordis & Howard, 1987; Chu & Berg, 1985).

As for pADX-OV, 20 μg of DNA was cotransfected into a 100-mm dish with 1 μg of pOV-Ref DNA (internal control) and cultured in the medium containing 25 μM forskolin. Six hours later, RNA was extracted from the cells (Ahlgren et al., 1990) and analyzed by primer extension or S1 nuclease protection assays. Human hepatocyte (HepG2) and monkey kidney (COS 1) cell lines were cultured, maintained, and treated in the same manner as described for Y1 cells.

Primer Extension Analysis. RNA was extracted from the cells with 6 M guanidine thiocyanate as described (Chirgwin et al., 1979). Poly(A)$^+$ RNA was further isolated through an oligo(dT)–cellulose column. Total RNA (30 μg) or poly(A)$^+$ RNA (5–10 μg) was then hybridized with 0.2 pmol of ^{32}P-labeled primer (5×10^5 cpm) in 10 mM Tris-HCl (pH 8)–1 mM EDTA containing 300 mM KCl at the appropriate annealing temperature depending on each primer. The locations of the primers are shown by the arrows in Figure 1B. The hybridized primer was extended by 50 units of MMLV-reverse transcriptase at 42 °C for 1 h in 45 μL of 40 mM Tris-HCl (pH 8.3), 125 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM each of dATP, dTTP, dCTP, and dGTP. In the cases when total RNA was used as template, 5 μL of TE and 12.5 μL of 0.5 M NaOH were added to terminate the reaction followed by boiling for 3 min. After removal to ice, the reaction mixture was neutralized by the addition of 12.5 μL each of 0.5 M HCl and 1 M Tris-HCl (pH 7.4). The extended products were precipitated by ethanol and analyzed by electrophoresis on a 6% polyacrylamide–7.5 M urea sequencing gel.

S1 Nuclease Mapping Analyses. Cytoplasmic RNA was prepared as described (Lund et al., 1990). Y1 cells were harvested by trypsin–EDTA treatment. They were washed once with phosphate-buffered saline, suspended and lysed by vortexing in 0.4 mL of 10 mM Tris-HCl (pH 8.6), 140 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40, and kept on ice for 5 min. After centrifugation at 15000*g* for 5 min, the postnuclear supernatants were incubated with 20 μL of 20% SDS and 10 μL of proteinase K (20 mg/mL) for 45 min at 37 °C. The samples were extracted with phenol–chloroform twice, and the cytoplasmic RNA was precipitated with ethanol. Quantitation of β-globin transcripts was carried out by S1 protection assay as described by Westin et al. (1987). RNA (20–30 μg) was hybridized with a 5′-end-labeled oligonucleotide probe which is complementary to the coding strand of β-globin gene from +75 to −18. The hybridization mixture was treated with 75 units of S1 nuclease for 30 min at 37 °C. The protected probes were analyzed following electrophoresis on a regular 10% polyacrylamide–7.5 M urea gel or a 6% polyacrylamide–7.5 M urea sequencing gel. After electrophoresis, the gel was fixed in 10% methanol–10% acetic acid prior to being dried. An autoradiogram was prepared from the dried gel, and radioactive bands were then excised from the gene and subjected to liquid scintillation counting.

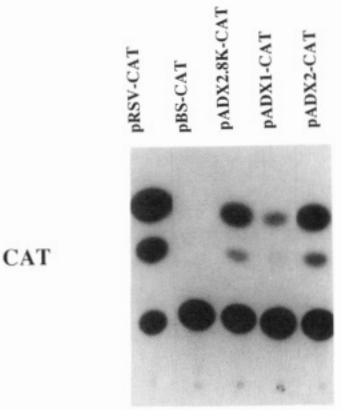

FIGURE 2: Both adrenodoxin 5′-flanking and intron 1 fragments direct efficient expression of the linked CAT structural gene in Y1 cells. Mouse adrenocortical Y1 tumor cells were transfected with various recombinant CAT plasmids for 24 h, and cell lysates were prepared, and transient CAT activity was assayed. pRSV-CAT carrying the Rous sarcoma viral promoter in front of the CAT structural gene serves as a positive control while the promoterless pBS-CAT serves as a negative control.

RESULTS

Bovine Adrenodoxin Gene Contains Two Separate, Functional Promoters. To define the promoter sequences in the bovine adrenodoxin gene, pADX2.8K-CAT was initially constructed and transfected into the mouse adrenocortical Y1 tumor cells. Plasmid pADX2.8K-CAT contains a 2.8-kb adrenodoxin DNA fragment including a portion of the 5′-flanking sequence, exon 1, intron 1, and a small region of exon 2 and thus the 5′-end of each of the two mRNA species (Figure 1A). As shown in Figure 2, the 2.8-kb adrenodoxin fragment supports efficient expression of the linked CAT gene, suggesting the presence of active promoter sequence(s). The constitutively expressed CAT activity derived from cells transfected with pADX2.8K-CAT was about 10% of the positive control pRSV-CAT, which contains the strong Rous sarcoma viral promoter sequence linked to the CAT structural gene. Under the same conditions, the promoterless pBS-CAT which contains only the CAT structural gene in the Bluescript vector gave rise to extremely low CAT activity. To examine whether the two mRNA species from the bovine adrenodoxin gene arise from two separate promoters, two smaller ADX-CAT constructs, pADX1-CAT and pADX2-CAT, were generated, each containing the 5′-origin of one of the two mRNA species reported previously (Kagimoto et al., 1988). As can be seen in Figure 1A, plasmid pADX1-CAT contains a 0.75-kb-long 5′-flanking region of the adrenodoxin gene while plasmid pADX2-CAT contains a 0.56-kb adrenodoxin fragment encompassing part of exon 1, intron 1, and a small portion of exon 2. Transient expression assays showed that the CAT gene was constitutively expressed under the control of each of these two adrenodoxin DNA fragments (Figure 2), indicating the presence of two separate promoters in the bovine adrenodoxin gene, one in the 5′-flanking region and one in intron 1. In addition, the CAT activity derived from pADX2-CAT is about 5–10-fold greater than that derived

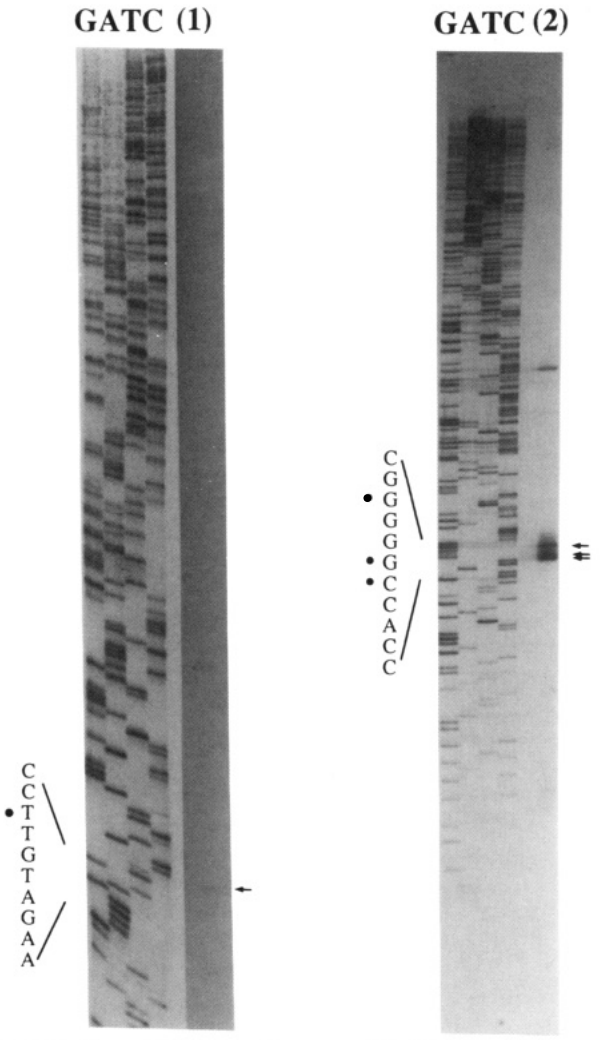

FIGURE 3: Correct initiation sites were utilized for transient transcription of the reporter gene driven by adrenodoxin promoters. Y1 cells were transfected with the recombinant plasmids (1) pADXP1-OVEC and (2) pADXP2-OVEC and incubated in the presence of 25 μM forskolin for 6 h. RNA was isolated and hybridized with specific primers for reverse transcriptase which bind to the positions indicated by the arrows in Figure 1B. Sequencing ladders derived from the same primers are shown at the left of the primer extension lanes. In order to visualize the primer extension products derived from pADXP1-OVEC, an overexposed lane was utilized which is properly aligned to the original sequence ladder obtained from a lighter exposure. The correct primer extension products are indicated by arrows, and the positions of transcription initiation are indicated by dots.

from pADX1-CAT, indicating that the promoter in the adrenodoxin intron 1 sequence, which is designated as ADXP2, is more efficient than the one in 5′-flanking sequence, which is designated as ADXP1.

Bovine Adrenodoxin Promoters Direct the Transcription of Reporter Gene at the Correct Initiation Sites. To determine whether the expression of the reporter gene was driven by the adrenodoxin promoters and the transcripts were initiated at the proper sites, we analyzed the transcription products of the recombinant genes by primer extension. Due to the difficulties of studying the readily degradable CAT mRNA, we constructed recombinant pADX-OV plasmids for this purpose, pADXP1-OV and pADXP2-OV. Upon primer extension, total RNA from Y1 cells transfected with pADXP2-OV produced major products about 112 nucleotides long (Figure 3, lane 2), corresponding to the length predicted for utilization of the ADXP2 promoter. On the other hand, no signal was observed with total RNA isolated from cells transfected with pADXP1-OV (data not shown). However, when 10 μg of poly(A)$^+$ RNA prepared from the pADXP1-OV-transfected cells was used as template, we were able to recognize a faint primer extension product 67 nucleotides long corresponding to the utilization of ADXP1 as promoter (Figure 3, lane 1). A second band 35-nt longer was also observed in some but not all poly(A)$^+$ RNA preparations. These results indicate that each of the bovine adrenodoxin promoters functions in directing the expression of the linked reporter gene in the transient expression system and that the positions of transcription start sites of the fusion genes correspond to the transcription start sites of the adrenodoxin gene.

Unique cAMP-Responsive Sequences Lie Upstream from Each Adrenodoxin Promoter. As the synthesis of adrenodoxin in bovine adrenocortical cells is regulated by ACTH via the action of cAMP, we examined the effect of cAMP on the function of each adrenodoxin promoter in the transient expression system. Y1 cells were transfected with recombinant pADXP1-OV and pADXP2-OV DNA and incubated in the presence and absence of 25 μM forskolin. Forskolin activates adenylate cyclase and thus increases the level of intracellular cAMP. The effect of forskolin on the expression of the pADX-OV fusion genes was detected by S1 nuclease mapping analyses of the transcripts generated in the transfected cells using a synthetic 93-mer oligonucleotide which is complementary to the coding strand of the β-globin gene from sequence +75 to −18 with two noncomplementary nucleotides present in the five nucleotides at the 3′-end (Westin et al., 1987). When adrenodoxin promoters were inserted 13 bases upstream from the β-globin structural gene and utilized to initiate the transcription (Figure 1B), a protected fragment 88 nucleotides long was observed. As shown in Figure 4A, Y1 cells transfected with the promoterless parent vector pOV-3 gave detectable β-globin transcripts only upon forskolin treatment. Plasmids pADXP1-OV and pADXP2-OV were constructed by fusing the adrenodoxin DNA fragments −678/+72 and +249/+808 to the β-globin structural gene, respectively. Therefore, each pADX-OV contains one of the adrenodoxin promoters as designated in the name. Plasmid pADXP1-OV which contains the weak adrenodoxin promoter reproducibly gave a pattern similar to that of pOV-3. In contrast, the pADXP2-OV fusion gene containing the stronger adrenodoxin promoter gave rise to a 5−10-fold greater basal expression which is further enhanced by forskolin (Figure 4A), suggesting the presence of a cAMP-responsive sequence associated with this promoter. The level of 88-mer does not completely reflect the correctly initiated transcripts since it also includes the products derived from any read-through transcripts. However, as shown by primer extension analysis (Figure 3, lane 2), the major population of transcripts are derived from the correct initiation sites.

Since ADXP1 is such a weak promoter, it is possible that the cAMP responsiveness is so weak that it is hard to detect even when the promoter is associated with a cAMP-responsive element. In order to further examine whether a cAMP-responsive element is present in the 5′-flanking region upstream of the ADXP1 promoter, we utilized another chimeric reporter gene system which adopts a heterologous test promoter. Such a chimeric system has been employed to identify the cAMP-responsive sequences in the bovine $P450_{17\alpha}$ (Lund et al., 1990), $P450_{SCC}$ (Ahlgren et al., 1990), and $P450_{c21}$ (Kagawa & Waterman, 1990, 1991) genes by ligating each DNA fragment to the β-globin minimal promoter and structural gene followed by transient transfection assays. Adrenodoxin 5′-flanking and intron 1 sequences immediately upstream from the promoter regions were generated by polymerase chain reactions and

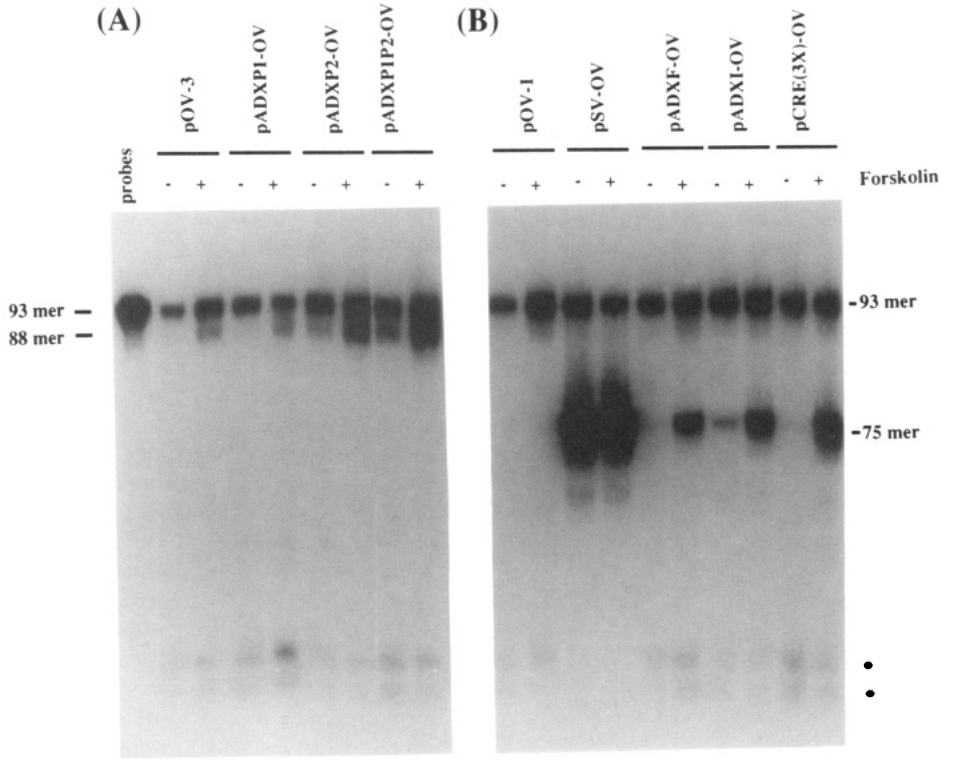

FIGURE 4: cAMP stimulates transient expression of the reporter gene in the pADX-OV plasmids. Mouse Y1 cells were transfected with various recombinant OVEC plasmids and incubated in the presence and absence of 25 μM forskolin for 6 h. Cytoplasmic RNA was isolated and analyzed by S1 nuclease protection assay followed by electrophoresis on a 6% polyacrylamide–7.5 M urea sequencing gel. The left-hand lane represents the radiolabeled 93-mer oligonucleotide probe used in S1 nuclease protection assays. The amount of protected fragments indicated as 88-mer and 75-mer represents the populations of transcripts derived under the control of (A) adrenodoxin promoter and (B) β-globin promoter, respectively. The asterisks indicate the positions of the protected fragments derived from the internal control pOV-Ref.

fused to the rabbit β-globin minimal promoter and its structural gene to generate pADXF-OV and pADXI-OV constructs (Figure 1C). In this case, since transcription was driven by the β-globin promoter, a protected 75-nucleotide fragment was detected (Figure 4B). The positive control pSV-OV in which the SV40 enhancer was inserted upstream of the minimal β-globin promoter yielded a high level of β-globin transcripts which was not increased by forskolin treatment, and the negative control pOV-1 gives rise to a very low level of globin transcripts in either case. However, when the cells were transfected with pCRE(3X)-OV which contains three head-to-tail repeats of the consensus cAMP-responsive element (CRE) derived from the human chorionic gonadotropin-α gene cloned into pOV-1 (Deutsch et al., 1987; Lund et al., 1990), a 10–20-fold induction of β-globin transcription was observed upon forskolin treatment. As shown in Figure 4B, both pADXF-OV and pADXI-OV yielded similar patterns of cAMP-enhanced transcription upon forskolin treatment. These results indicate that intron 1 in the bovine adrenodoxin gene contains a cAMP-responsive sequence which promotes transcription derived from the nearby ADXP2 promoter and the 5′-flanking region of the adrenodoxin gene contains a second cAMP-responsive sequence which is clearly functional with a heterologous promoter but which cannot be demonstrated to be functional with its homologous ADXP1 promoter. The sequence present in the intron 1 region near the ADXP2 promoter is designated as CRS2 (*c*AMP-*r*esponsive *s*equence 2) while that located in the 5′-flanking region upstream the ADXP1 promoter is designated as CRS1.

The interactions of these two cAMP-responsive sequences with both promoters upon cAMP stimulation were further investigated by transfection of Y1 cells with the recombinant plasmid pADXP1P2-OVEC which contains both adrenodoxin promoters and their respective CRS. The cells were then cultured in the presence and absence of 25 μM forskolin for 6 h before RNA was isolated and analyzed. As shown in Figure 4A, the cAMP-induced transcription observed with pADXP1P2-OV was 3–5-fold higher than that from pADXP2-OV. Further examination of the transcripts derived from each individual promoter revealed that the majority of the transcripts were derived from ADXP2 as shown in Figure 5. The amount of transcript derived from ADXP1 was very low. CRS1 may exert a stimulating effect on the transcription derived from the ADXP2 promoter in the pADXP1P2-OV construct, since elevated cAMP-induced transcription was observed with this construct as compared to that of pADXP2-OV; however, the context of these two promoters is altered by the deletion indicated in Figure 1B, and thus a synergistic relationship between these two promoters remains unclear. It is not possible to determine whether CRS2 exerts a similar effect on the ADXP1 promoter since ADXP1 is such a weak promoter.

Cycloheximide Does Not Block cAMP-Stimulated Transcription in the Transient Expression Systems. We have previously reported that adrenodoxin mRNA (Okamura et al., 1985) as well as the mRNAs derived from $P450_{17\alpha}$ (Zuber et al., 1986), $P450_{scc}$ (John et al., 1984), and $P450_{c21}$ (John et al., 1986a) genes are enhanced in bovine adrenocortical cells upon ACTH or cAMP treatment but this mRNA accumulation is blocked by the protein synthesis inhibitor cycloheximide (John et al., 1986b). However, recent studies on the cAMP-responsive elements associated with the P450 genes have indicated that the cAMP-enhanced transcription of the linked reporter genes is not sensitive to the action of cycloheximide in the transient expression system when β-globin promoter was utilized for transcription (Lund et al., 1990; Ahlgren et al., 1990; Kagawa & Waterman, 1990). We therefore tested the effect of cycloheximide on expression

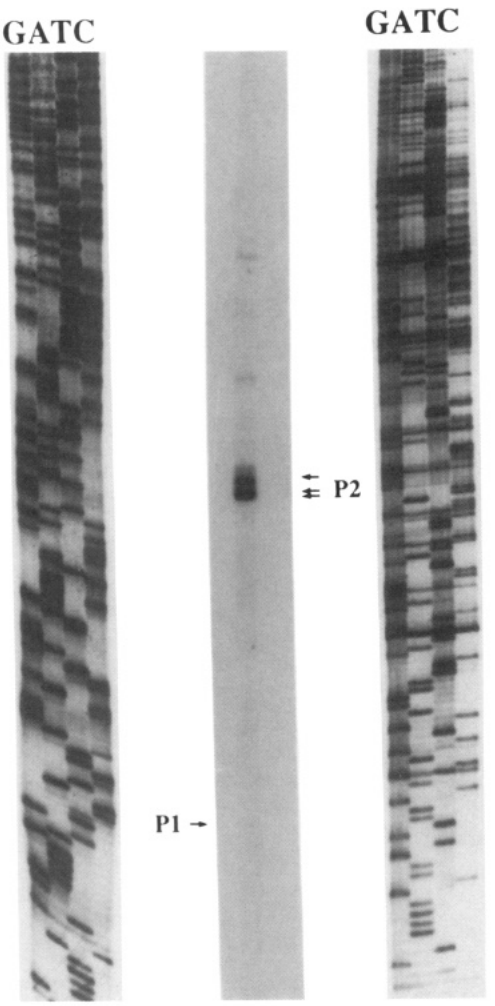

FIGURE 5: Primer extension analysis of transcripts derived from pADXP1P2-OVEC. Y1 cells were transfected with recombinant plasmid pADXP1P2-OVEC and cultured in the presence of 25 μM forskolin for 6 h. Poly(A)$^+$ RNA was isolated and analyzed by primer extension analysis utilizing specific primers as indicated by the arrows in Figure 1B. The primer extension products which represent the populations of correctly initiated transcripts are indicated as P1 and P2. Sequencing ladders derived from the specific primers to pADXP1-OVEC (left panel) and pADXP2-OVEC (right panel) were also shown to indicate the positions of initiation sites.

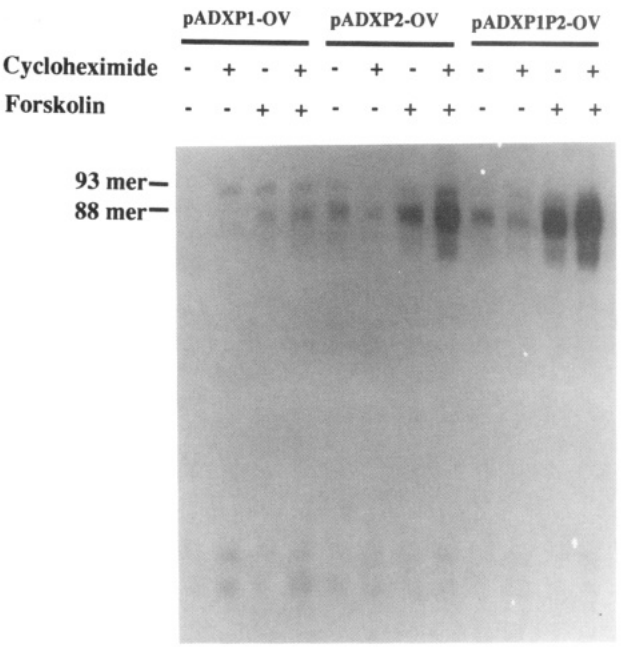

FIGURE 6: Cycloheximide does not inhibit the cAMP-stimulated transient expression of the reporter gene. Y1 cells were transfected by various pADX-OVEC plasmids and incubated in the presence or absence of 25 μM forskolin in combination with or without 40 μM cycloheximide. After 6 h, RNA was isolated and analyzed by S1 nuclease protection assays. The protected fragments (88-mer) were analyzed by a 6% polyacrylamide–7.5 M urea sequencing gel.

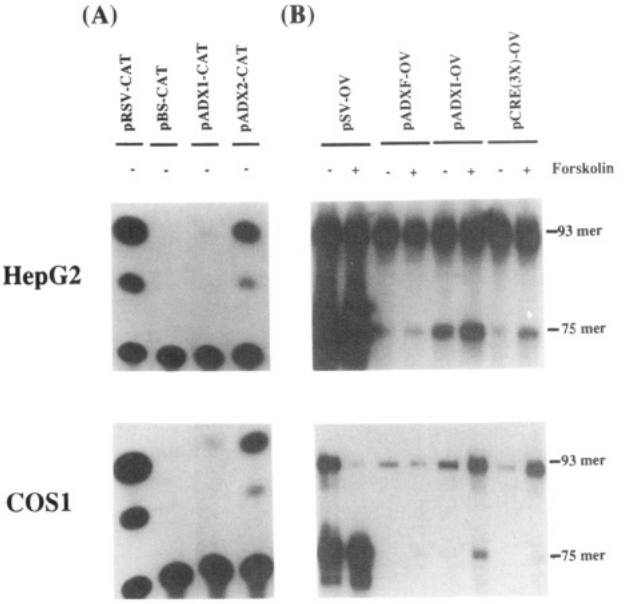

FIGURE 7: Adrenodoxin promoters and cAMP-responsive sequences function in liver and kidney cells. HepG2 (upper panel) and COS1 (bottom panel) cells were transfected with various recombinant CAT and OVEC plasmids and incubated in the presence and absence of 25 μM forskolin as indicated. Transient expression was assayed by monitoring (A) the CAT activity and (B) the transcripts generated in the transfected cells.

derived from the β-globin promoter under the influence of the two adrenodoxin cAMP-responsive elements. After transfection with pADXF-OV and pADXI-OV DNA, respectively, Y1 cells were maintained in the presence and absence of 25 μM forskolin in combination with or without 40 μM cycloheximide. S1 mapping analyses of transcripts indicated that the cAMP-enhanced transcription derived from the β-globin promoter was not inhibited by cycloheximide (data not shown). We then tested the cycloheximide effect on expression derived from pADXP1-OVEC and pADXP2-OVEC constructs, in which the cAMP-responsive elements are present in their native context associated with their respective adrenodoxin promoters. As can be seen in Figure 6, cycloheximide alone had little inhibitory effect on the basal expression of the reporter gene. When added concomitantly with forskolin, cycloheximide does not block the cAMP-stimulated transcription but instead it promotes the stimulation of expression. As measured by [^{35}S]methionine incorporation, cycloheximide inhibited protein synthesis under these conditions by more than 95%. This effect of superinduction by cycloheximide on cAMP-stimulated expression of the reporter gene under the influence of adrenodoxin promoters could be duplicated by incubating the cells with 200 μM puromycin (data not shown).

Adrenodoxin Gene Functions in Liver and Kidney Cells. Since adrenodoxin is known to be expressed in liver and kidney, the effect of adrenodoxin fragments on the expression of β-globin gene expression was examined in hepatocyte (HepG2) and kidney (COS1) cell lines. As can be seen in Figure 7A, both promoters appear to be active in directing the expression of the linked CAT structural gene, albeit at much lower rates as compared to Y1 cells. However, the same pattern of pro-

moter strength is followed, with ADXP2 being the stronger promoter. In both cell types, the adrenodoxin CRS1 and CRS2 would confer cAMP responsiveness to the heterologous β-globin promoter (Figure 7B), but not to their respective adrenodoxin promoters (data not shown).

Discussion

Transient transfection of cultured cells with chimeric plasmid constructs containing different reporter genes linked to the DNA fragments from either the 5′-flanking region or intron 1 of the bovine adrenodoxin gene has demonstrated that this gene contains two promoters. Examination of the RNA isolated from cells transiently transfected with the chimeric genes showed that the primer extension products correspond to the 5′-ends of the isolated adrenodoxin mRNAs. Thus, it is now clear that the two species of bovine adrenodoxin mRNA (Okamura et al., 1987) arise from transcription initiated by these two promoters, one in the 5′-flanking region (ADXP1) and the other in intron 1 (ADXP2). The expression in mouse adrenocortical Y1 tumor cells under the control of ADXP2 is about 5–10-fold higher than that of ADXP1. The fact that ADXP2 serves as a stronger promoter correlates with our earlier results from Northern analyses of steady-state RNA levels in bovine adrenocortical cells in which the adrenodoxin transcripts containing the sequences in exon 2 represent the major species while those containing exon 1 represent the minor species (Okamura et al., 1987).

It is not clear why there should be two separate promoters for the bovine adrenodoxin gene, especially since the translation of the mRNA derived from ADXP1 would lead to the synthesis of only a 14 amino acid peptide due to the presence of a stop codon in exon 1 (Kagimoto et al., 1988; Sagara et al., 1990). To investigate the mechanisms regulating the expression derived from these two promoters, we have found that two cAMP-responsive elements may be associated with this gene. The adrenodoxin intron 1 region immediately upstream from the ADXP2 promoter confers the cAMP responsiveness to the nearby adrenodoxin promoter as well as to the heterologous β-globin promoter in directing the expression of the linked β-globin structural gene. The upstream 0.75-kb-long 5′-flanking region also confers the cAMP responsiveness to the heterologous β-globin minimal promoter but not to its native ADXP1 promoter. Our earlier results from Northern blot analysis indicated that levels of both the major and minor forms of adrenodoxin mRNA were enhanced by cAMP in primary cultures of bovine adrenocortical cells. The present results clearly indicate that CRS2 plays a role in enhancing the transcription of the major mRNA species. However, since CRS1 only functioned to enhance transcription in response to cAMP with a heterologous promoter (β-globin) and not with the homologous ADXP1 promoter, we can only conclude that CRS1 has the potential to enhance transcription of the minor form of adrenodoxin mRNA in response to cAMP. Whether CRS1 functions in the native gene is not established by these studies.

We have mapped the detailed sequences of the two cAMP-responsive elements identified in this report by deletional analyses (data not shown). As shown in Figure 8, neither one of the cAMP-responsive sequences shares sequence homology to the consensus *c*AMP-*r*esponse *e*lement (CRE) (5′-TGACGTCA-3′) (Roesler et al., 1988) nor to each other. Furthermore, neither CRS1 nor CRS2 in the bovine adrenodoxin gene shows significant sequence homology to the unique CRS sequences identified in this laboratory in the human CYP21B gene (Kagawa & Waterman, 1990) or the bovine CYP17 (Lund et al., 1990) and CYP11A (Ahlgren et al., 1990) genes. However, the higher level of cAMP-stimulated transcription observed when the heterologous β-globin promoter was utilized rather than the homologous adrenodoxin promoter may arise due to a different spatial display around the promoter regions which leads to more efficient interactions of the core promoter elements (RNA polymerase II and TFIID, -IIA, -IIB, and -IIE) and other transcriptional factors (e.g., cAMP-responsive sequence binding proteins) when using the β-globin promoter.

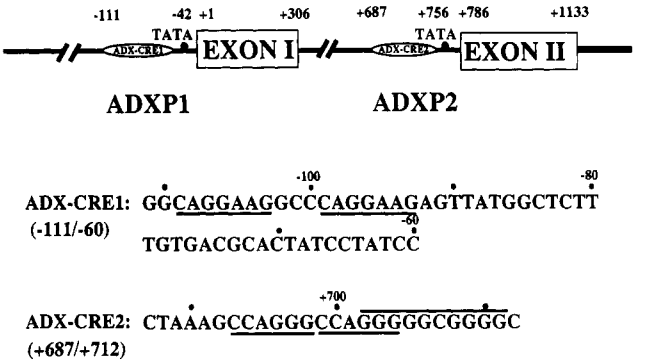

FIGURE 8: Schematic diagram of the 5′-end of the bovine adrenodoxin gene and the sequences of the regions containing cAMP-responsive elements. Two 7 and 6 bp repeats (underlined) were found in ADX-CRE1 and ADX-CRE2, respectively. A GC box was located in ADX-CRE2 which is overlined. Dots are placed at 10-nucleotide intervals in the two sequences.

A number of eukaryotic gene have been demonstrated to have multiple transcription start sites driven by separate promoters, for example, the *Drosophila* ADH gene (Corbin & Maniatis, 1989; Benyajati et al., 1983), the mouse α-amylase gene (Schibler et al., 1983), the myosin light chain 1 and 3 coding genes (Periasamy et al., 1984), and the yeast invertase gene SUC2 (Carlson & Botstein, 1982). In all of these cases, the separate promoters specify unique tissue-specific or developmentally timed programs of expression from the alternate start site. Currently, we are examining the physiological roles of the two adrenodoxin promoters as well as the two cAMP-responsive sequences in terms of their involvement in either tissue-specific or developmentally regulated expression of adrenodoxin gene. As described herein, these two promoters work less efficiently in the cells derived from nonsteroidogenic tissues which are known to express low levels of adrenodoxin, such as hepatocyte (HepG2 and Chang) and kidney (COS1) cells. However, the same pattern of promoter strength is followed, with ADXP2 being the stronger in all cell types examined. In the hepatocyte and kidney cells, the two cAMP-responsive elements identified confer cAMP responsiveness to the heterologous β-globin promoter, respectively, but we cannot detect this effect on their own respective adrenodoxin promoters. It is not clear whether this is because both promoters function weakly in the nonsteroidogenic tissue-derived cells or whether the cAMP responsiveness of adrenodoxin expression is tissue-specific.

Accumulation of adrenodoxin mRNA in primary bovine adrenal cells in response to ACTH or cAMP has been shown to be inhibited by cycloheximide (John et al., 1986b). However, upon transient transfection of Y1 cells with the chimeric pADX-OV, cycloheximide has no inhibitory effect on forskolin-stimulated reporter gene transcription. Superinduction of the reporter gene was observed when cells were treated with cAMP concomitantly with cycloheximide regardless of whether the adrenodoxin promoter or the β-globin promoter was utilized. Similar phenomena were observed with the β-globin promoter in the studies of the cAMP-responsive sequences

present in the bovine $P450_{17\alpha}$ (Lund et al., 1990), $P450_{SCC}$ (Ahlgren et al., 1990), and $P450_{c21}$ (Kagawa & Waterman, 1990) genes. Thus, the cycloheximide inhibition of transcription is not associated with the promoter. Further investigation is required to understand this reproducible discrepancy between studies of the complete adrenodoxin gene in vivo and transient expression studies using fragments of this gene.

References

Ahlgren, R., Simpson, E. R., Waterman, M. R., & Lund, J. (1990) *J. Biol. Chem. 265*, 3313–3319.

Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., & Russell, D. W. (1989) *J. Biol. Chem. 264*, 8222–8229.

Benyajati, C., Spoerel, N., Haymerle, H., & Ashburner, M. (1983) *Cell 33*, 125–133.

Carlson, M., & Botstein, D. (1982) *Cell 28*, 145–154.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chu, G., & Berg, P. (1985) *Nucleic Acids Res. 13*, 2921–2930.

Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E., & Goodman, H. M. (1986) *Nature 323*, 353–356.

Corbin, V., & Maniatis, T. (1989) *Genes Dev. 3*, 2191–2200.

Deutsch, P. J., Jameson, J. L., & Habener, J. F. (1987) *J. Biol. Chem. 262*, 12169–12174.

Driscoll, W. J., & Omdahl, J. L. (1986) *J. Biol. Chem. 261*, 4122–4125.

Fordis, C. M., & Howard, B. H. (1987) *Methods Enzymol. 151*, 382–397.

Graham, F. L., & van der Eb, A. J. (1973) *Virology 52*, 456.

John, M. E., John, M. C., Ashley, P., MacDonald, R. J., Simpson, E. R., & Waterman, M. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5628–5632.

John, M. E., Okamura, T., Dee, A., Adler, B., John, M. C., White, P. C., Simpson, E. R., & Waterman, M. R. (1986a) *Biochemistry 25*, 2846–2853.

John, M. E., John, M. C., Boggaram, V., Simpson, E. R., & Waterman, E. R. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4715–4719.

Kagawa, N., & Waterman, M. R. (1990) *J. Biol. Chem. 265*, 11299–11305.

Kagawa, N., & Waterman, M. R. (1991) *J. Biol. Chem. 266*, 11199–11204.

Kagimoto, M., Kagimoto, K., Simpson, E. R., & Waterman, M. R. (1988) *J. Biol. Chem. 263*, 8925–8928.

Kramer, R. E., DuBois, R. N., Simpson, E. R., Anderson, C. M., Kashiwagi, K., Lambeth, J. D., Jefcoate, C. R., & Waterman, M. R. (1982) *Arch. Biochem. Biophys. 215*, 478–485.

Kruse, F., Komro, C. T., Michnoff, C. H., & MacDonald, R. J. (1988) *Mol. Cell. Biol. 8*, 893–902.

Le Goascogne, C., Robel, P., Gonezou, M., Sananes, N., Baulieu, E. E., & Waterman, M. R. (1987) *Science 237*, 1212–1215.

Lund, J., Ahlgren, R., Wu, D., Kagimoto, M., Simpson, E. R., & Waterman, M. R. (1990) *J. Biol. Chem. 265*, 3304–3312.

Matocha, M. F., & Waterman, M. R. (1984) *J. Biol. Chem. 259*, 8672–8678.

Matocha, M. F., & Waterman, M. R. (1985) *J. Biol. Chem. 260*, 12259–12265.

Okamura, T., John, M. E., Zuber, M. X., Simpson, E. R., & Waterman, M. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5705–5709.

Okamura, T., Kagimoto, M., Simpson, E. R., & Waterman, M. R. (1987) *J. Biol. Chem. 262*, 10335–10338.

Pedersen, J. I., Oftebro, H., & Vanngard, T. (1977) *Biochem. Biophys. Res. Commun. 76*, 666–673.

Periasamy, M., Strehler, E. E., Garfinkel, L. I., Gubits, R. M., Roesler, W. J., Vandenbark, G. R., & Hanson, R. W. (1988) *J. Biol. Chem. 263*, 4063–4066.

Raza, H., & Avadhani, N. G. (1988) *J. Biol. Chem. 263*, 9533–9541.

Ruiz-Opazo, N., & Nadal-Ginard, B. (1984) *J. Biol. Chem. 259*, 13595–13604.

Sagara, Y., Sawae, H., Kimura, A., Sagara-Nakano, Y., Morohashi, K., Miyoshi, K., & Horiuchi, T. (1990) *J. Biochem. 107*, 77–83.

Schibler, U., Hagenbiichle, O., Wellaner, P. K., & Pittet, A. C. (1983) *Cell 33*, 501–508.

Waterman, M. R., & Simpson, E. R. (1989) *Recent Prog. Horm. Res. 45*, 533–566.

Westin, G., Gerster, T., Miiler, M. M., Schaffner, G., & Schaffner, W. (1987) *Nucleic Acids Res. 15*, 6787–6798.

Zuber, M. X., John, M. E., Okamura, T., Simpson, E. R., & Waterman, M. R. (1986) *J. Biol. Chem. 261*, 2475–2482.