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cAMP post-transcriptionally diminishes the abundance of adrenodoxin reductase mRNA

(steroid hormone/flavoprotein/electron transfer/RNA stability)

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ABSTRACT **Adrenodoxin reductase (AR; ferridoxin: $NADP^+$ oxidoreductase, EC 1.18.1.2) is a flavoprotein that mediates electron transport from NADPH to all known mitochondrial forms of cytochrome P450. AR mRNA was found in all human adult and fetal tissues examined; however, it was vastly more abundant in tissues that synthesize steroid hormones. The ratio of the 18^- form of mRNA lacking 18 alternately spliced bases to the 18^+ form was ≈100:1 and remained constant irrespective of the tissue or hormonal manipulation, indicating that the alternate splicing is a passive nonregulated event. AR protein was unchanged by forskolin treatment of human JEG-3 cytotrophoblast cells for 24 h, but the mRNA diminished. Phorbol 12-myristate 13-acetate and cycloheximide had no effect, even though these agents had the expected effects on P450scc and adrenodoxin mRNAs. cAMP decreased the abundance of AR mRNA expressed from both transfected plasmids and the endogenous gene, indicating the effect was post-transcriptional. AR gene transcription in JEG-3 cells and promoter–chloramphenicol acetyltransferase constructs transfected into JEG-3 cells were unresponsive to forskolin. Powerful basal transcription elements were identified between −46 and −214 bases from the principal transcriptional initiation site, a region containing six elements closely resembling the binding site for transcription factor SP1.**

The first and rate-limiting step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone by mitochondrial cytochrome P450scc. Enzymatic activity requires electrons from NADPH that are transferred to P450scc by the flavoprotein adrenodoxin reductase (AR; ferridoxin-:$NADP^+$ oxidoreductase, EC 1.18.1.2) and the iron–sulfur protein adrenodoxin, which mediate electron transport to all known mitochondrial forms of cytochrome P450 (1). AR, which is loosely associated with the inner mitochondrial membrane (2), receives electrons from NADPH by way of a flavin moiety bound near its N terminus (3) and then passes them to adrenodoxin, which functions as an electron shuttle for all mitochondrial P450s (4, 5). By contrast, microsomal cytochrome P450 enzymes utilize a flavoprotein different from AR.

The human cDNAs and genes for P450scc and adrenodoxin have been cloned, and their regulated expression has been studied. A single species of P450scc mRNA (6) is encoded by a single gene (7) on chromosome 15q23–q24 (8) that is expressed primarily in steroidogenic tissues (6, 9). This mRNA is induced by tropic hormones acting by way of cAMP (9–11) by stimulating P450scc gene transcription (12). Three species of human adrenodoxin mRNAs that differ only in the lengths of their 3′ untranslated regions (13) are encoded by two genes (14, 15) on chromosome 11q22 (8, 16) that are expressed in all tissues examined (13). Adrenodoxin mRNA also accumulates in response to tropic hormones acting by way of cAMP (13, 17, 18), apparently through a post-transcriptional mechanism (S.T.B. and W.L.M., unpublished data).

We have cloned the cDNA (19) and gene (20) on chromosome 17q24–q25 (8) for human AR. There are two species of human AR mRNA that differ by 18 bases encoding the sequence ALLLCQ (amino acids 204–209) of the longer form (19, 20). The function, if any, of the 18^+ form is unclear, as the six extra amino acids would probably disrupt the second β-sheet of the NADPH binding site (20). We have now studied the abundance, tissue distribution, and hormonal regulation of the two forms of AR mRNA, and we have characterized the cis elements of the promoter associated with tissue-preferential expression.

MATERIALS AND METHODS

JEG-3 cells were grown as described (13) with or without 40 μM forskolin, 1 mM 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), 30 nM phorbol 12-myristate 13-acetate (PMA), or cycloheximide (20 μg/ml). JEG-3 RNA was prepared as described (21) or by direct lysis of the cell monolayer with 0.5% SDS (22).

Plasmid phtAR contains the full-length 1.9-kilobase AR cDNA in pUC18, including the alternately spliced 18-base region lying within a 178-base-pair (bp) *Rsa* I fragment (19). Plasmid phaAR contains the 18^- form of the cDNA, lacking the 18-base region and part of the 5′ end (19). Plasmid p9 contains the 178-bp *Rsa* I fragment of phtAR cloned into pBKS+. Plasmid pE-AR^+ is vector pECE (23) expressing the long 18^+ form of AR mRNA; plasmid pE-AR^-, expressing the full-length 18^- form, was constructed by replacing the *Dra* III–*Xba* I fragment of phtAR with the *Dra* III–*Xba* I fragment of phaAR.

A 932-bp *Bam*HI–*Nco* I fragment of the human AR gene from bases −904 to +28 was isolated, the ATG translational start site was removed by mung bean nuclease digestion, and the 932-bp *Bam*HI–blunt *Nco* I fragment was cloned into pACAT (21), to yield pAR-904. Plasmids pAR-527, pAR-217, and pAR-46 were constructed by internal deletions of pAR-904. Transfection and chloramphenicol acetyltransferase (CAT) assays were done as described (21).

RNA probes were prepared from p9 transcribed with T7 RNA polymerase and used for RNase protection assays (12, 24). The 259-base RNA probe from p9 protects a 178-bp fragment from AR 18^+ mRNA and two fragments of 128 bp and 32 bp from AR 18^- RNA. The 385-base lamin probe contains a 285-bp *Pst* I fragment of lamin A cDNA (25) and protects a 285-base band from endogenously produced lamin and a 78-base band from a 1.9-kilobase lamin cDNA fragment expressed in pECE (pElam).

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Abbreviations: AR, adrenodoxin reductase; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate.

Antibodies to AR and adrenodoxin were raised by C. R. Wolf (ICRF Molecular Pharmacology Group, Edinburgh) against the proteins expressed in *Escherichia coli*. Purified human adrenodoxin (26) was kindly provided by L. Vickery. (University of California, Irvine). Immunoblot analysis was done as described (27).

RESULTS

Tissue Distribution of the Two Forms of AR mRNA. Based on the number of 18^+ and 18^- form cDNAs identified, this laboratory suggested (19) that the 18^+ form represented 10–20% of AR mRNA. To determine this proportion more accurately and to determine whether there were tissue-specific differences in this proportion that might suggest different functions for the two mRNAs, we used a sensitive RNase protection assay. The 18^+ mRNA protects the 178-base fragment and the 18^- mRNA protects fragments of 128 and 32 bases. All human RNA samples examined contained AR mRNA, consistent with its role as a generic electron transport protein for all mitochondrial forms of cytochrome P450. AR mRNA was most abundant in fetal and adult adrenal and was present at ≈10% of the adrenal level in adult and fetal testis and fetal ovary (Fig. 1). AR mRNA was present at much lower levels in term placenta and fetal liver, kidney, lung, brain, muscle, and heart, at ≈1% of the adrenal level. In all tissues, the overwhelming majority of the AR was of the 18^- form. The 178-base band corresponding to the 18^+ form was detected only in the adrenal and testis, where the ratio of the 18^- to 18^+ form was ≈100:1. However, very long exposures of this and other films suggest there are small amounts of the 18^+ mRNA in all tissues.

Hormonal Repression of AR mRNA in JEG-3 Cells. The mRNAs for P450scc, P450c17, P450c21, and adrenodoxin increase in response to cAMP stimulation in various species and cell types (1, 28), reaching maximal accumulation after 36–48 h (9, 13, 18, 29). Yet AR mRNA was induced minimally in human granulosa cells when P450scc and adrenodoxin mRNAs were maximal (19). As it seemed logical that increased synthesis of P450scc and adrenodoxin would not be fully effective without a concomitant increase in AR, we examined the responses of AR mRNA to short-term incubation of JEG-3 cells with 1 mM 8-Br-cAMP, or 40 μM forskolin. Both cAMP (Fig. 2) and forskolin (data not shown) decreased the abundance of the 18^- form of AR mRNA (128-base protected fragment) over a 12-h period. The reduction was detectable after ≈5 h, and by 12 h the abundance of AR mRNA was reduced to <50% of control. Longer exposures of these autoradiographs showed the same effect on the 178-bp protected fragment (18^+ form of AR mRNA), consistent with the hypothesis that the appearance of the 18^+ form is a passive nonregulated event. Nuclear lamin mRNA in the same cells showed a slight 2-fold increase, consistent with the known mild inductive effect of cAMP on lamin mRNA. Similarly, the abundance of adrenodoxin mRNA in these same cells increased ≈4-fold with 12 h of stimulation (data not shown), consistent with our observations in JEG-3 cells (13) and in primary cultures of human ovarian granulosa, adrenal, and testicular cells (18). Thus the cAMP-induced reduction in AR mRNA is specific to that mRNA and does not reflect a global effect on the cells.

Since both cAMP and forskolin, which stimulate the protein kinase A system, reduced the abundance of AR mRNA, we examined the effects of the phorbol ester PMA, which stimulates the protein kinase C system. Cells were incubated with 40 μM forskolin, 30 nM PMA, or both for 3, 6, 12, 18, or 24 h (Fig. 3). No effect of PMA on AR mRNA was seen; PMA plus forskolin had the same effect as forskolin alone. In this experiment, the forskolin-induced reduction in AR mRNA is not as prominent as in Fig. 2; however, it is clear

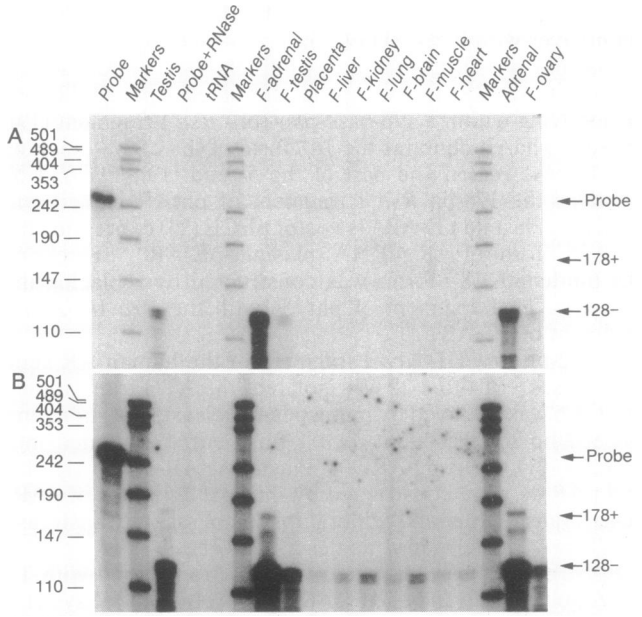

FIG. 1. Tissue distribution of AR mRNA. (*A*) RNase protection experiment. Molecular size markers are *Hpa* II fragments of pUC 18. The 259-bp probe seen in the probe lane is totally digested by RNase A in the probe + RNase lane. Other lanes show incubation of the probe with 20 μg of RNA from the various human tissues indicated, except for adult and fetal testis where 10 μg was used. Protection of the 18^+ form of AR mRNA is indicated by the arrow designated 178+ and protection of the 18^- form is indicated by the arrow designated 128−. The 32-base fragment protected by the 18^- form is run off the gel. The autoradiogram was exposed for 12 h. (*B*) Same experiment after autoradiography for 7 days.

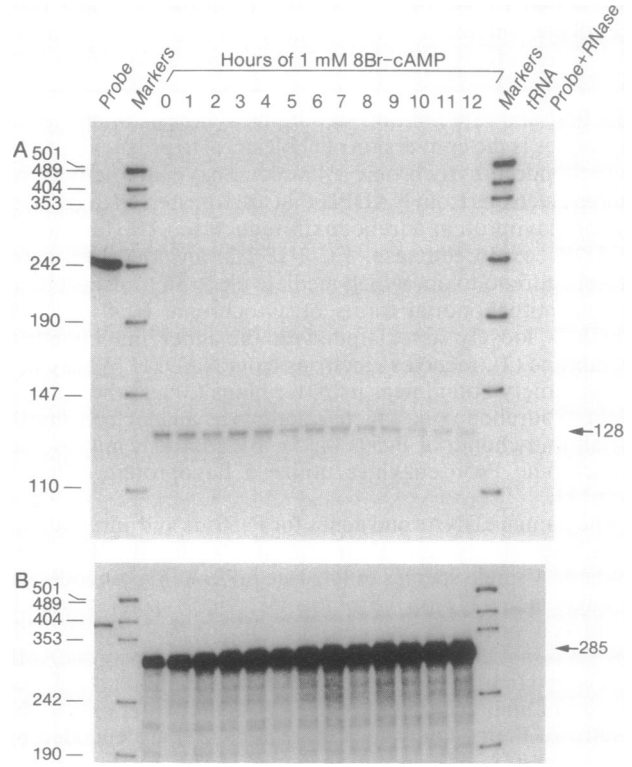

FIG. 2. Effect of 8-Br-cAMP or forskolin on AR mRNA in JEG-3 cells. Each lane contains 10 μg of JEG-3 cell RNA. (*A*) Diminution of the 18^- form of AR mRNA (arrow at 128 bases) in JEG-3 cells incubated for 0–12 h with 1 mM 8-Br-cAMP. (*B*) Induction of nuclear lamin mRNA (arrow at 285 bases) in the same JEG-3 cells.

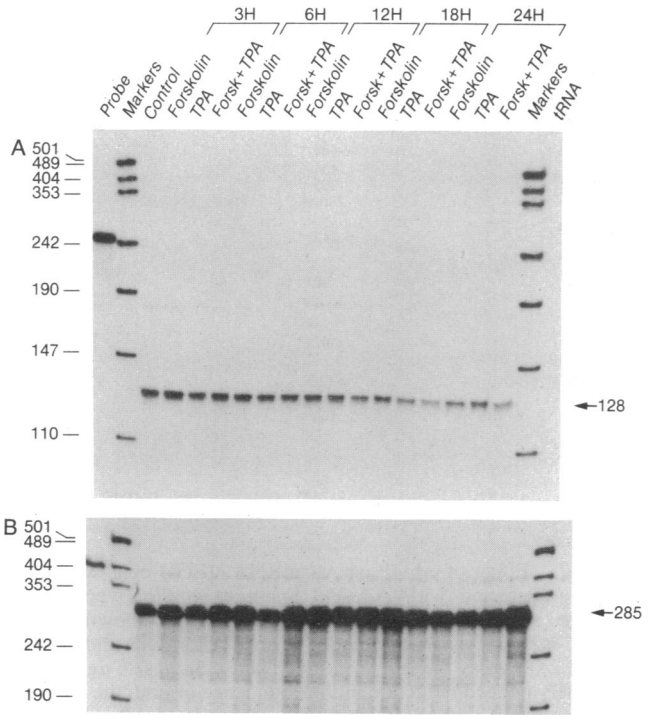

FIG. 3. Effect of phorbol ester (PMA, termed TPA in the lane designations) on AR mRNA in JEG-3 cells. Each lane contains 20 μg of JEG-3 cell RNA. (*A*) Lack of effect of 30 nM PMA, with or without 40 μM forskolin on the 18⁻ form of AR mRNA (arrow at 128 bases). JEG-3 cells were incubated with the various drugs for 3, 6, 12, 18, or 24 h, as shown. (*B*) Effect of PMA and forskolin on nuclear lamin (arrow at 285 bases) in the same cells studied in *A*.

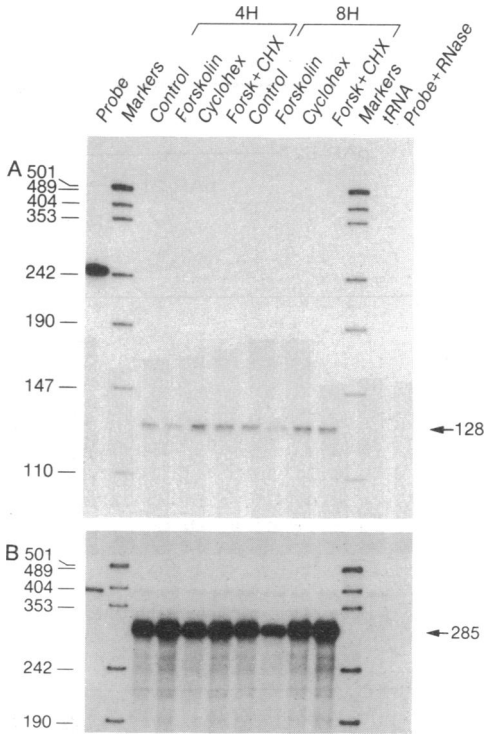

FIG. 4. Effect of cycloheximide (CHX) on AR mRNA. Each lane contains 20 μg of RNA from JEG-3 cells incubated for 4 or 8 h (as shown) with no added drugs (control), 40 μM forskolin (Forsk), cycloheximide (20 μg/ml), or both. (*A*) Effects on the 18⁻ form of AR mRNA (arrow at 128 bases). (*B*) Effects on nuclear lamin mRNA in the same cells (arrow at 285 bases).

the forskolin was active, as the lamin mRNA in the same cells increased up to 2-fold in response to forskolin, consistent with the response of lamin to cAMP (Fig. 2). PMA also slightly induced lamin mRNA in JEG-3 cells (Fig. 3*B*).

Relationship of Protein Synthesis to AR mRNA Accumulation. The role of protein synthesis in the induction of steroid hormone synthesis has been studied in many systems, but its effects on AR are unknown. We blocked protein synthesis with cycloheximide and examined its effects on AR mRNA in JEG-3 cells treated with and without forskolin for 4 or 8 h (Fig. 4). Cycloheximide slightly increased AR mRNA abundance compared to control or cells treated with forskolin alone at both 4 and 8 h, and the combination of cycloheximide and forskolin resulted in a slight diminution in the abundance of adrenodoxin mRNA seen with cycloheximide alone. Thus inhibition of protein synthesis has minimal effects on AR mRNA. Similarly, cycloheximide had no discernible effect on nuclear lamin mRNA in the same cells.

Correlation Between the Induction of AR mRNA and Protein. To determine if the forskolin-induced diminution in AR mRNA had acute effects on AR protein, we examined mitochondrial proteins by immunoblot analysis. A single species of AR of ≈50 kDa was seen (Fig. 5). This could be consistent with translation of both adrenodoxin mRNAs, as the 0.75-kDa difference in the 18⁺ and 18⁻ forms would not be detectable. Treatment of JEG-3 cells with 40 μM forskolin for up to 24 h had no effect on AR. By contrast, the single 13-kDa form of adrenodoxin increased ≈4- to 5-fold in the same cells.

Effects of Forskolin on AR Gene Transcription. Initial nuclear run-on assays showed that forskolin induced transcription of the P450scc gene 2- to 3-fold after 2–12 h of incubation but had no effect on AR (data not shown). To examine the apparently constitutive expression of the human AR gene in greater detail, we examined the behavior of its promoter (Fig. 6). DNA fragments from +28 to −46, −214, −527, and −904 bases from the principal transcriptional start site were cloned into pACAT (21), to yield plasmids pAR-46, pAR-214, pAR-527, and pAR-904. When JEG-3 cells transfected with pAR-904 were treated with forskolin for 0–12 h, CAT activity expressed by the AR promoter was 80–100 times greater than the promoterless pACAT control and was not altered by forskolin. By contrast, the cAMP-regulated P450scc construction pAnscc-267 (12) expresses basal activity ≈5 times greater than promoterless pACAT and is induced 6-fold by forskolin but is still only one-third as active as basal pAR-904 expression. To determine where this powerful promoter activity lies, we examined smaller fragments of the AR promoter. There was little difference among the

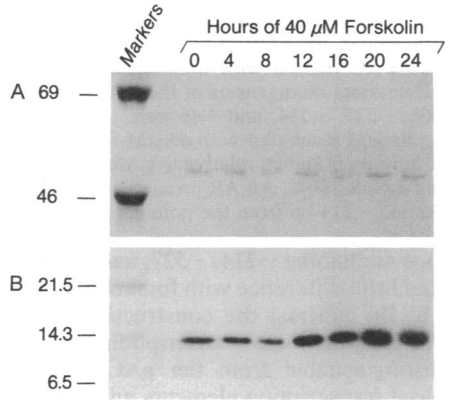

FIG. 5. Effect of forskolin on AR protein in JEG-3 cells. Immunoblot analysis of 50-μg samples of total mitochondrial proteins from JEG-3 cells incubated with 40 μM forskolin for the times indicated. (*A*) Blot probed with rabbit antibody to human AR expressed in *E. coli*. (*B*) Blot probed with antibody to purified human adrenal adrenodoxin.

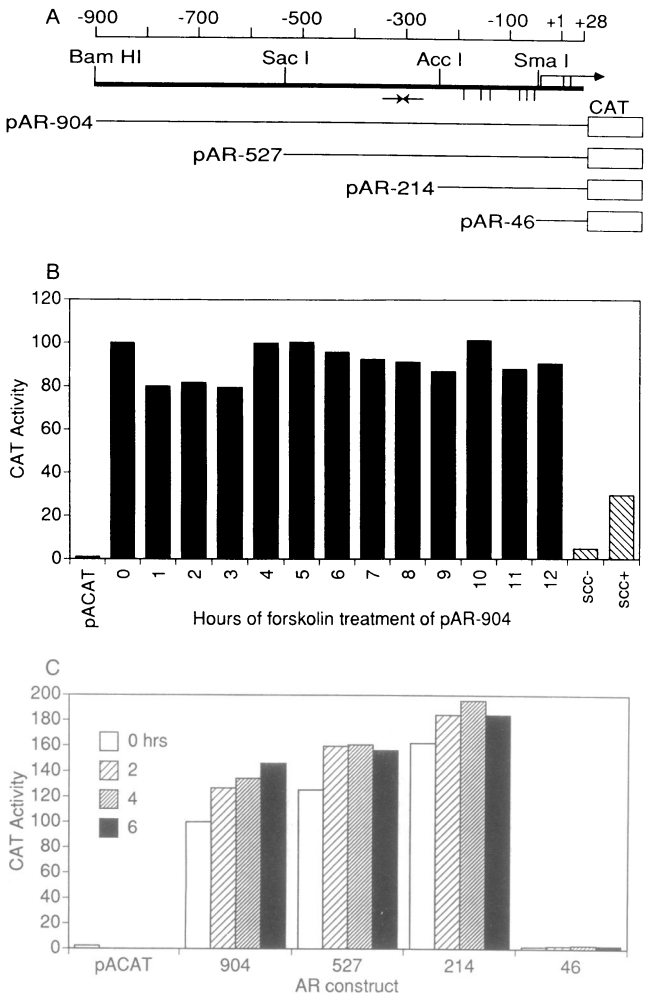

FIG. 6. Activity of AR promoter fragments transiently transfected into JEG-3 cells. (*A*) Diagram of the human AR gene promoter. Upper bar, scale in bp; thick bar, landmarks in the DNA sequences used. The three principal transcriptional initiation sites at positions +7, +1, and −46 are shown by the rightward arrow; the six GGGCGGG sequences are shown by lines below the bar at positions −50, −68, −80, −140, −159, and −190. The inverted repeat is shown by two head-to-head arrows. The four CAT constructions using the restriction sites indicated on the bar are shown below. (*B*) CAT activity transcribed from pAR-904 in JEG-3 cells treated with 40 μM forskolin. CAT activity is shown relative to untreated cells (0 h of forskolin = 100%). Control plasmid pAnscc-267 containing 267 bases of the human P450scc gene promoter is shown after incubation with and without forskolin for 12 h. JEG-3 cells transiently transfected with pAR-904 were treated with forskolin for the various times shown. (*C*) Deletional mutagenesis of the AR gene promoter. Plasmids pAR-904, −527, −214, and −46 were transiently transfected into JEG-3 cells and incubated with 40 μM forskolin for the times shown. CAT activity is shown relative to pAR-904 without forskolin treatment and equals 100%. All AR promoter activity appears to lie between −46 and −214 bp from the nominal cap site.

constructions containing −214, −527, and −904 bases of the promoter and little difference with forskolin incubation for 0, 2, 4, or 6 h. By contrast the construction containing −46 bases of the promoter was transcriptionally inert, yielding values indistinguishable from the pACAT control. Thus powerful basal transcription elements appear to lie between −46 and −214 bases of the human AR promoter.

Effect of cAMP on AR mRNA. Both forskolin and cAMP reduced the abundance of AR mRNA in JEG-3 cells (Fig. 2). However, no effect of forskolin or cAMP was seen on total AR protein (Fig. 5) or on AR gene transcription (Fig. 6). Therefore, we sought to examine the effect of cAMP on the

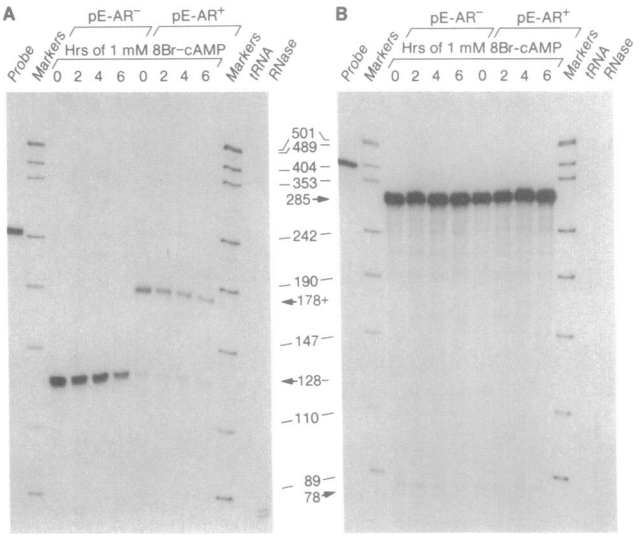

FIG. 7. Effect of cAMP on AR mRNA. JEG-3 cells were transiently transfected with AR and lamin expression vectors and incubated for 0–6 h with 1 mM 8-Br-cAMP as shown. Vector pE-AR⁻ is pECE expressing the 18⁻ form of AR mRNA and pE-AR⁺ is pECE expressing the 18⁺ form. Each lane contains 20 μg of RNA. (*A*) cAMP-induced reduction in the abundance of both the 18⁻ form (128-base band) and 18⁺ form (178-base band) of AR mRNA expressed from the transfected plasmids. The endogenous 18⁻ form of AR mRNA can be seen in the cells transfected with the 18⁺ vector. (*B*) No effect is seen on nuclear lamin mRNA expressed from the cotransfected lamin expression vector pElam (78-base band) in RNA from the same cells shown in *A*. The endogenous lamin mRNA is seen as the 285-base protected fragment.

half-life of AR mRNA. Initial experiments using actinomycin D to block transcription of the AR gene suggested that AR mRNA has a half-life of >24 h; however, experiments with actinomycin D plus cAMP gave variable results (data not shown). Therefore, we examined the effect of cAMP on AR mRNA transcribed from a transfected cDNA expression vector not regulated by cAMP (Fig. 7). JEG-3 cells were transfected with plasmid pECE containing either the AR 18⁻ cDNA (pE-AR⁻) or the 18⁺ cDNA (pE-AR⁺). The abundance of both the 18⁻ and 18⁺ forms of RNA expressed by the transfected vector was reduced to ≈50% of control after 6 h of incubation with 1 mM 8-Br-cAMP. No 18⁺ RNA (178-base fragment) is seen in the RNA from cells transfected with pE-AR⁻, but the abundant endogenous 18⁻ form RNA is seen in cells transfected with pE-AR⁺. As an internal control, the cells were simultaneously transfected with pElam expressing nuclear lamin cDNA. The resulting vector-expressed lamin mRNA was not affected by the 8-Br-cAMP (78-base band), indicating that transcription from the pECE vector is insensitive to cAMP. The endogenous lamin mRNA in these cells is seen as the 285-base band, showing minimal induction of the endogenously produced lamin mRNA after 6 h of 8-Br-cAMP, consistent with the slow response of lamin mRNA to 8-Br-cAMP seen in Figs. 2 and 3.

DISCUSSION

The mRNA for AR, like that for adrenodoxin (13), is found in all tissues examined. Furthermore, both AR (Fig. 1) and adrenodoxin (13) mRNAs are substantially more abundant in steroidogenic tissues, indicating strong concordance with the tissue distribution of P450scc mRNA. The low abundance of AR mRNA in placenta is not surprising. Although the placenta is a steroidogenic organ, the abundance of its steroidogenic enzyme mRNAs is diluted by its large mass; even in the steroidogenically very active midterm (25 week) placenta, the abundance of P450scc (6) and adrenodoxin (13) mRNAs was

low; the placental RNA used in Fig. 1 was derived from a term (40 week) placenta, which is steroidogenically much less active. The high abundance of AR mRNA in the fetal ovary is somewhat surprising. The fetal ovary is steroidogenically quiescent and has virtually no mRNA for P450scc or P450c17 between 15 and 21 weeks of gestation (30). Yet during the same period of gestation, the fetal ovary has readily detectable adrenodoxin (18) and AR (Fig. 1) mRNA. The very low abundance of the 18^+ form of the mRNA and the apparently constant 100:1 ratio of 18^- to 18^+ forms suggest that the 18^+ mRNA arises as a passive phenomenon in the splicing of any AR mRNA precursor, irrespective of the cell type in which it is transcribed. A lack of an active role for the 18^+ mRNA would be consistent with the hypothesis that the additional encoded ALLLCQ sequence would disrupt the NADPH binding site (20). Thus the abundance of adrenodoxin mRNA is regulated in a tissue-specific fashion, but its alternate splicing is not.

Blocking protein synthesis with cycloheximide has various effects on steroidogenesis in various systems. Early experiments *in vivo* suggested that ongoing protein synthesis was required for stimulation of steroidogenesis by corticotropin; furthermore, cycloheximide substantially inhibits cAMP-induced mRNA accumulation and gene transcription for P450scc, adrenodoxin, P450c11, P450c17, and P450c21 in primary cultures of bovine adrenocortical cells (31). However, P450scc gene transcription in mouse Leydig MA-10 cells is insensitive to cycloheximide (32), and P450scc mRNA is unaffected whereas adrenodoxin mRNA increases in human granulosa cells treated with cycloheximide (17). In JEG-3 cells (13) and in primary cultures of human cytotrophoblasts (29), cycloheximide abolishes P450scc mRNA accumulation as it does in bovine adrenal cells but increases adrenodoxin mRNA accumulation (as it does in granulosa cells). Cycloheximide had a very slight inductive effect on AR mRNA, but this effect was minimal compared to the profound changes seen in P450scc and adrenodoxin mRNAs in JEG-3 cells and other systems. Cycloheximide also blocked the cAMP-induced reduction in AR mRNA; a requirement for ongoing protein synthesis is a common feature of many mRNA destabilizing systems (33). Thus, as described above, cycloheximide can have profound inductive or inhibitory effects on mRNAs for steroidogenic enzymes, or, as shown here, it may have very little effect, depending on the enzyme and cell type.

Unlike the stimulatory effects of cAMP and forskolin seen on the mRNAs for P450scc, adrenodoxin, and other steroidogenic enzymes (9, 11, 12, 18, 21), both cAMP and forskolin diminished the abundance of AR mRNA in JEG-3 cells. This effect appears to result from a cAMP-induced shortening of AR mRNA half-life, as shown by the effects of cAMP on AR mRNA produced from transfected expression vectors. Both the RNA polymerase run-on assays and the AR promoter transfection experiments showed no effects of cAMP or forskolin on AR gene transcription in JEG-3 cells.

The AR gene has multiple transcriptional start sites, with most transcription initiated from sites at +7, +1, and −46 bp with respect to the principal site at +1 (20). The upstream DNA is notable for the lack of TATA and CAAT sequences, for six SP-1 sites (GGGCGGG; consensus sequence KKGGCGKRY, where K is G or T, R is G or A, and Y is C or T) with the C centered at positions −50, −68, −80, −140, −159, and −190 and for a pair of inverted repeats at positions −297 to −304 and −319 to −326 that could form a stem–loop structure with a ΔG of −10.2 kcal/mol (1 cal = 4.184 J) at 25°C (20). The pAR-214 construction deletes this potential stem–loop structure but retains all six SP-1 sites; this construction has all the promoter activity seen in pAR-904 (or more). Thus SP1 may be an important factor in regulating AR promoter activity. Nevertheless, the 100-fold difference in the abundance of AR mRNA in adrenal vs. nonsteroidogenic tissues indicates there is significant tissue preference, if not tissue-specificity, of expression.

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