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Expression of murine 21-hydroxylase in mouse adrenal glands and in transfected Y1 adrenocortical tumor cells

(steroid biosynthesis/corticotropin regulation/DNA-mediated gene transfer/major histocompatibility complex)

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ABSTRACT **The *S* region of the murine major histocompatibility complex contains two structurally related genes (*21-OHase A* and *21-OHase B*) that encode 21-hydroxylase (21-OHase), an enzyme essential for the synthesis of adrenal steroids. Expression of these two genes has been analyzed by using oligonucleotide probes specific for the *21-OHase A* and *B* genes and by DNA-mediated gene transfer. Hybridization of the oligonucleotides to blots of BALB/c adrenal RNA demonstrated that all 21-OHase mRNA is derived from the *21-OHase A* gene. Cosmids bearing either the *21-OHase A* or *B* gene were introduced into Y1 adrenocortical tumor cells by cotransfection with pSV2-neo. Cells transfected with the *21-OHase A* gene expressed 21-OHase as determined by steroid metabolism and by RNA blot hybridization; 21-OHase transcripts were not detected in parent Y1 cells or in cells transfected with the *21-OHase B* gene. Treatment of *21-OHase A* transfectants with adrenocorticotropin increased 21-OHase mRNA levels by up to 10-fold, thus mimicking the observed effect of this hormone on 21-OHase levels in primary adrenal cultures. The regulated expression of the *21-OHase A* gene in transfected Y1 cells should provide a useful system for the investigation of factors controlling the adrenal-specific regulation of 21-OHase activity.**

Adrenal steroid biosynthesis requires the action of four distinct cytochrome P-450 enzymes; these enzymes include cholesterol side-chain-cleavage enzyme, 11β-hydroxylase, 17α-hydroxylase, and 21-hydroxylase (21-OHase) (1). Adrenal 21-OHase has a molecular weight of 52,000, requires an NADPH-dependent cytochrome reductase (2), and is primarily responsible for the unique properties of the adrenocortical steroids. A deficiency of 21-OHase is the most common cause of congenital adrenal hyperplasia in man (3). Corticotropin (ACTH) regulates the activity of 21-OHase by increasing its synthesis (4). ACTH also regulates the transcription of the other three adrenal cytochrome P-450 enzymes and cAMP has been implicated as a mediator in this regulation (5–7).

The development of recombinant DNA probes specific for the adrenal steroid hydroxylating enzymes has accelerated the investigation of the regulation of their expression. Both the human (8) and murine (9) *21-OHase* genes have been characterized by using a bovine cDNA probe. These studies have defined two *21-OHase* genes within a duplicated segment of the class 3 region of the human and murine major histocompatibilty complexes. The murine genes lie approximately 4 kilobases (kb) 3′ of the genes for the murine sex-limited protein and fourth component of complement (C4) as shown in Fig. 1. The human *21-OHase* genes are in a corresponding position 3′ of the duplicated human C4 genes. By using analyses of restriction fragment polymorphisms of genomic DNA from 21-OHase-deficient patients, White *et al.* (10) proposed that the human *21-OHase B* gene was responsible for all of the adrenal 21-OHase enzymatic activity, whereas the *21-OHase A* gene was functionally inactive. The difficulties in molecular analysis of human subjects precluded the definition of the basis for lack of *21-OHase A* expression.

The availability of inbred mouse strains, the Y1 adrenocortical cell line that expresses many of the enzymes involved in adrenal steroidogenesis (11), and cloned genomic fragments containing the entire *21-OHase A* and *B* genes (9) affords an excellent system in which to study the genetics and regulation of expression of the *21-OHase* genes. In this study, we utilized *21-OHase A*- and *B*-specific oligonucleotide probes to show that only the *21-OHase A* transcript is present in BALB/c adrenal RNA. When cosmid clones containing the intact *21-OHase A* and *B* genes were used to transfect the Y1 murine adrenocortical tumor cell line, only the cells transfected with cosmids containing *21-OHase A* expressed 21-OHase mRNA and enzymatic activity. These results suggest that only the *21-OHase A* gene from the BALB/c mouse is active. Furthermore, transcription of the *21-OHase* gene in Y1 cell transfectants was increased following treatment with ACTH, suggesting that the transfected gene retained the elements required for hormonal regulation.

MATERIALS AND METHODS

Materials. Restriction and modification enzymes were obtained from New England Biolabs and used according to the manufacturer's instructions. Media, serum supplements, antibiotics, and geneticin (G418) were from GIBCO. BALB/c mice (6- to 8-weeks old) were purchased from The Jackson Laboratory. [γ-^{32}P]ATP, [α-^{32}P]CTP, and [1,2-^{3}H]progesterone were from New England Nuclear. The 15-base-long oligonucleotides were synthesized by the solid-phase phosphoramidite technique using an Applied Biosystems (Foster City, CA) 380A synthesizer and purified by elution from a 20% (wt/vol) polyacrylamide preparative gel. Silica gel for thin layer chromatography (silica gel 60 GF_{254}) was purchased from Brinkman.

Cell Culture and DNA-Mediated Gene Transfer. Mouse Y1 adrenocortical tumor cells were maintained as monolayers in 150-mm plastic dishes with F-10 medium supplemented with 15% (vol/vol) heat-inactivated horse serum, 2.5% (vol/vol) heat-inactivated fetal bovine serum, penicillin (200 units/ml), and streptomycin sulfate (280 μg/ml). Gene transfer was

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Abbreviations: 21-OHase, 21-hydroxylase; ACTH, corticotropin; kb, kilobase(s).

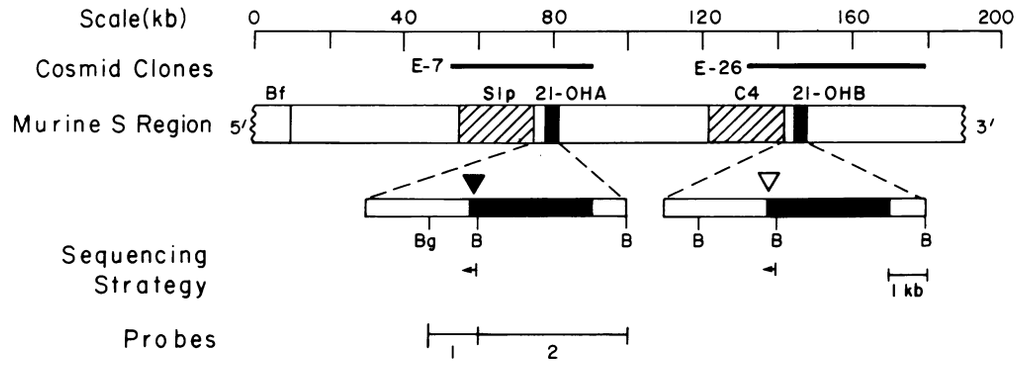

FIG. 1. Molecular map of the murine *S* region. The relative locations and orientations of *S* region genes are shown. Heavy bars indicate positions of the E-7 and E-26 cosmids used in the transfection experiments. *S* region segments that encode the *21-OHase A* and *B* genes have been expanded below. The positions of the oligonucleotide probes specific for *21-OHase A* (▼) and *21-OHase B* (▽) are indicated above the inserts. Restriction sites for *Bam*HI (B) and *Bgl* II (Bg) are shown relative to the *21-OHase A* and *B* genes. The locations for E-7:1.3 (probe 1) and E-7:3.8 (probe 2) are indicated. The small arrows indicate the strategy used in sequencing the 5′ regions of the *21-OHase* genes. Slp, sex-limited protein; C4, the fourth component of complement; *21-OH A* and *B*, *21-OHase A* and *B*.

essentially as described (12, 13). Cosmid DNA (2 μg) was mixed with 2 μg of pSV2-neo. These DNAs were incorporated into a calcium phosphate precipitate and applied to 150-mm culture dishes containing 5×10^6 cells in MEM Alpha supplemented as above. Cells were cultured in the transfection medium for 24 hr, and then the medium was replaced with supplemented F-10 medium. After an additional 24 hr of culture, selection was initiated with G418 at 100 μg of active form/ml and continued for 2 wk. Survivors were then cloned and cultured in selective medium. ACTH regulation experiments were performed by incubating Y1 cells and *21-OHase A* transfectants in ACTH at 5 milliunits/ml for 24 hr.

Nucleic Acid Analyses. High molecular weight DNA and total cellular RNA were prepared (14, 15) and Southern and RNA blot analyses were performed as described (16, 17). Nucleic acids bound to nitrocellulose were hybridized with ^{32}P-labeled oligonucleotides by using standard conditions (18). Oligonucleotide hybridizations were performed at 37°C for 18 hr in 0.9 M NaCl/0.09 M sodium citrate/1× Denhardt's solution/100 μg of yeast tRNA/ml/0.1% $NaDodSO_4$. Oligonucleotide probes were used at a final concentration of 0.4 ng/ml. Filters were washed for two 30-min periods at 22°C with 0.9 M NaCl/0.09 M sodium citrate/0.1% sodium pyrophosphate and then for two 30-min periods at 41°C with the same solution. Restriction fragments of *21-OHase A* and *B* were inserted into the phage M13 vector mp9 (19) and sequenced by the dideoxy chain-termination technique (20).

Bioassay of 21-OHase Enzymatic Activity. Y1 adrenocortical tumor cells and G418-resistant transformants were cultured in 16-mm multiwell dishes in F-10 medium without G418 for 4–7 days. Cells were then incubated in 1 ml of fresh medium containing [1,2-^{3}H]progesterone (1 μCi; 1 Ci = 37 GBq) for 24 hr at 36.5°C. At the end of the incubation the medium was removed, and 2 μg each of progesterone, 20α-hydroxyprogesterone, deoxycorticosterone, and corticosterone were added as carrier. Steroids were extracted into 5 vol of methylene chloride, washed with 1 ml of 0.1 M NaOH, and chromatographed on a thin layer of silica gel in chloroform acetone, 70:30 (vol/vol). Steroids were visualized by using a 254-nm UV lamp. The steroid-containing zones of silica gel, as well as the interzones, were scraped, and radioactivity in the corresponding fractions was determined by scintillation spectrometry.

RESULTS

Analysis of Expression of 21-OHase A and B mRNA Levels in BALB/c Adrenal Glands. The extreme homology between the *21-OHase A* and *B* genes precluded the isolation of restriction fragments that distinguished their gene products by differential hybridization. We, therefore, utilized the strategy shown in Fig. 1 to determine partial nucleotide sequences of the *21-OHase A* and *B* genes. The two genes differed by 5 nucleotides in 350 base pairs sequenced (unpublished data). These sequences were then used to synthesize specific 15-base oligonucleotide probes that differ by two bases, designated 21-OHase a and 21-OHase b, respectively. The sequences (21-OHase a: 5′ TTCCG*GAG*CTTCCAT 3′; 21-OHase b: 5′ TTCCG*CAA*CTTCCAT 3′) are complementary to the DNA sequences that encode amino acids 22–27 of the 21-OHase protein, based on approximately 70% homology of the predicted amino acid sequence with that reported for porcine 21-OHase (21). To confirm the appropriate hybridization specificity of the probes, Southern blots of *Bam*HI-digested plasmids E-7:6.7 and E-15:2.1, which contain the subcloned *21-OHase A* and *B* gene segments, respectively, were hybridized with nick-translated probe 1 or with kinase-labeled 21-OHase a or b oligonucleotides. The results (Fig. 2) clearly document appropriate hybridization of the oligonucleotides: the 21-OHase a probe hybridizes with the E-7:6.7 fragment but not with the E-15:2.1 fragment, whereas the reverse pattern is seen with the 21-OHase b oligonucleotide. Nick-translated probe 1, which contains a 1.3-kb *Bgl* II/*Bam*HI fragment derived from E-7, hybridizes with both fragments (data not shown).

The oligonucleotide probes were hybridized to blots of total adrenal RNAs from male and female BALB/c mice (Fig. 2*B*). RNA samples from both males and females showed hybridization of the 21-OHase a oligonucleotide with an RNA species of 2.2 kb, corresponding in size to 21-OHase mRNA. No signal was detected with the 21-OHase b oligonucleotide, even when the autoradiogram was exposed four times as long as shown in Fig. 2*B*. Similar results were obtained in two separate experiments utilizing different adrenal RNA preparations. Although the experiment shown in Fig. 2*B* suggests a higher level of expression of *21-OHase* in female than male mice, this has not been a consistent finding in other experiments using different RNA preparations.

These results clearly indicate a markedly disproportionate expression of the *21-OHase A* and *B* genes in BALB/c adrenals. Given the limitations of our assay system, we cannot exclude a low level of expression of the *21-OHase B* gene; however, densitometric scans of the autoradiograms indicate that this level is at most 5% that of the *21-OHase A* gene.

Analysis of 21-OHase Enzymatic Activity in Y1 Cells Transfected with *21-OHase A* and *B* Genes. G418-resistant clones of Y1 cells were recovered after transfection with

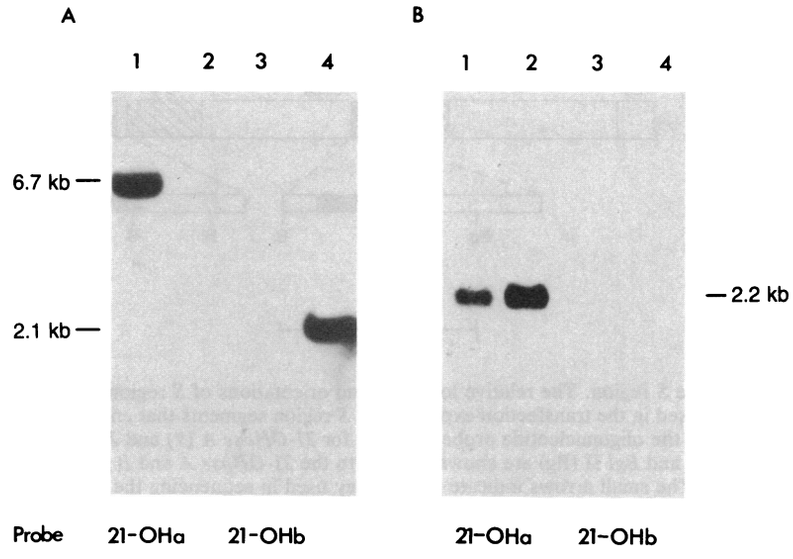

FIG. 2. Hybridization with *21-OHase A*- and *B*-specific oligonucleotide probes. (*A*) Demonstration of specific oligonucleotide hybridization. Plasmids E-7:6.7 and E-15:2.1, which contain the sequences found in the 21-OHase a and b oligonucleotides, respectively, were digested with *Bam*HI and analyzed by Southern blotting. Lanes 1 and 2, hybridization with the 21-OHase a oligonucleotide; lanes 3 and 4, hybridization with the 21-OHase b oligonucleotide. (*B*) Blot analysis of adrenal mRNA. Total RNA was isolated from adrenals of male and female BALB/c mice, and 15 μg of each RNA sample was analyzed by hybridization with the 21-OHase a and b oligonucleotides. Lanes 1 and 2, hybridization of male and female samples with the 21-OHase a oligonucleotide; lanes 3 and 4, hybridization of male and female samples with the 21-OHase b oligonucleotide. 21-OH a and b, 21-OHase a and b.

pSV2-neo and *21-OHase* cosmid DNAs. These clones were screened for 21-OHase activity by measuring the conversion of [1,2-^{3}H]progesterone to 21-hydroxylated products. In parental Y1 cells, the [^{3}H]progesterone was converted principally to the 11β,20α-dihydroxy derivative; less than 10% of the radioactivity was found in association with the corticosterone fraction (Fig. 3). The observations are consistent with reports (22–24) indicating that Y1 cells metabolize steroids to 11β,20α-dihydroxyprogesterone but do not produce 21-hydroxylated steroid products. Incubation of Y1 cells with [1,2-^{3}H]corticosterone for 24 hr resulted in the accumulation of more polar products (data not shown). Although these polar products have not been identified, they may have resulted at least in part from the conversion of corticosterone to the 20α-hydroxylated derivative (22).

Clones of Y1 adrenal cells isolated after transfection with the *21-OHase A* gene fell into three distinct categories (Fig. 3). Some clones, such as IC4, incorporated little radioactivity into the corticosterone fraction and appeared to be negative for 21-OHase activity. In this regard, these clones resembled the Y1 parent. Clones such as IIA1 converted [^{3}H]progesterone to a product which migrated with corticosterone. The amount of radioactivity in the corticosterone fraction was equal to that associated with 11β,20α-dihydroxyprogesterone, and more polar products did not accumulate. Thus, these clones were positive for 21-OHase activity. IIA3 and related clones also converted [^{3}H]progesterone to corticosterone as well as to more polar products. Inasmuch as the total amount of radioactivity in the corticosterone and more polar fractions exceeded the amount in the 11β,20α-dihydroxyprogesterone fraction (Fig. 3), these clones were defined as strongly positive for 21-OHase. Of 29 transfectants screened, 14 had 21-OHase activity by these criteria. Of these clones, 8 were strongly positive for 21-OHase activity.

Demonstration of 21-OHase mRNA in Transfected Y1 Cells. Total cellular RNAs from nontransfected Y1 cells and from transfectant clones were prepared and characterized by RNA blot analysis. No 21-OHase mRNA was detected in the nontransfected Y1 cells, but analyses of E-7 transfectants (Fig. 4*A*) demonstrated the presence of 21-OHase mRNA which comigrates with adrenal 21-OHase mRNA. An additional RNA species of approximately 4 kb is also detected in blots of RNA from adrenals and E-7 transfectants. Although this molecule has not been characterized fully, it most likely represents a precursor of the mature 21-OHase mRNA. Alternatively, it could result from aberrant processing of the 21-OHase transcript. A positive correlation was found be-

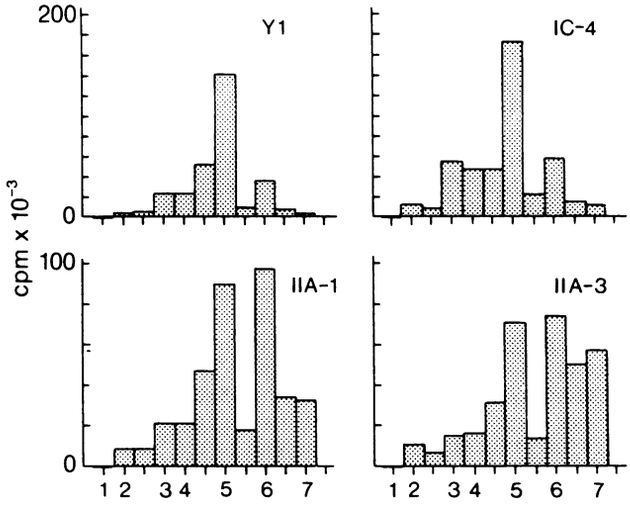

FIG. 3. Demonstration of 21-OHase activity in Y1 cells transfected with the *21-OHase A* gene. Y1 cells and clones transfected with E-7 DNA were incubated with [1,2-^{3}H]progesterone for 24 hr and steroid products were analyzed by thin layer chromatography on silica gel. Progesterone, 20α-hydroxyprogesterone, deoxycorticosterone, and corticosterone were identified on the basis of migration of external standards. Inasmuch as an external standard for 11β,20α-dihydroxyprogesterone was not available, this compound was identified on the basis of the migration of the major, UV-absorbing endogenous steroid produced by Y1 cells (22). Zones of silica gel containing the reference steroids as well as interzones were counted for distribution of radioactivity. The figure shows the relative positions of the standards on the gel: 1, front; 2, progesterone; 3, 20α-hydroxyprogesterone; 4, deoxycorticosterone; 5, 11β,20α-dihydroxyprogesterone; 6, corticosterone; and 7, origin.

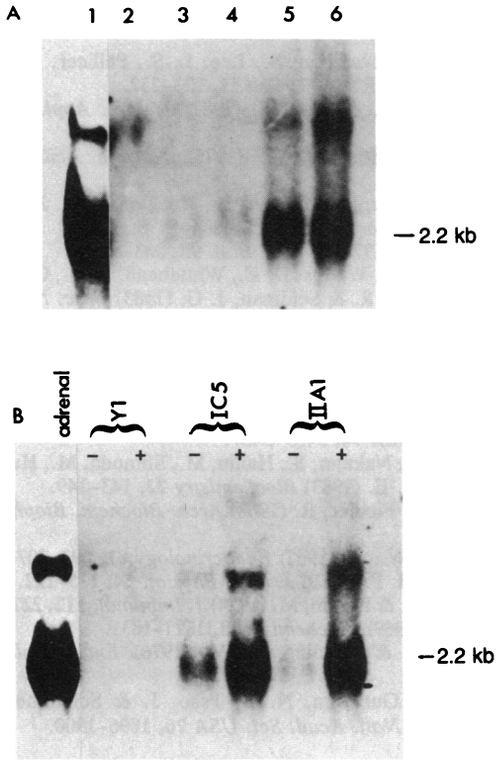

FIG. 4. RNA blot analysis of Y1 transfectants. (*A*) Demonstration of 21-OHase mRNA in E-7 transfectants. Total RNAs were isolated from untransfected Y1 cells and transfectant clones and analyzed by RNA blot hybridization. The filter was hybridized with nick-translated probe 2. Lanes: 1, 5 μg of BALB/c adrenal RNA; 2, 16 μg of Y1 RNA; 3, 16 μg of transfectant IC1 RNA; 4, 16 μg of transfectant IC4 RNA; 5, 8 μg of transfectant IC5 RNA; 6, 8 μg of transfectant IIA3 RNA. (*B*) ACTH regulation of *21-OHase* expression. Y1 cells and transfectant clones IC5 and IIA1 were incubated for 24 hr, with (+) or without (–) ACTH (5-milliunits/ml). Total RNAs were isolated and 15 μg of each sample and 5 μg of BALB/c adrenal RNA were analyzed by RNA blotting with nick-translated probe 2.

tween the 21-OHase mRNA levels and the 21-OHase metabolic activity described above (Fig. 3). Thus, transfectants IC5 and IIA3, which exhibit the highest functional activity, also have the strongest signals in blots.

21-OHase mRNA was not detected in seven E-26-transfected clones. These transfectant clones had between 3 and 10 additional copies of the *21-OHase B* gene as assessed by increases in the relative intensity of *21-OHase B*-related bands versus *21-OHase A*-related bands on Southern blot analyses (data not shown). Although cosmid E-7 also contains the gene for sex-limited protein, no expression of this gene was detected in RNA blot analyses of transfectants (data not shown).

ACTH Regulation of *21-OHase* Gene Expression. ACTH increases 21-OHase levels in primary adrenal cultures (4); we, therefore, examined its effect on 21-OHase mRNA in Y1 cells and E-7 transfectants. Nontransfected Y1 cells and transfectant clones IC5 and IIA1 were incubated for 24 hr in F-10 medium containing 5 milliunits of ACTH/ml. Total cellular RNAs were prepared and the amount of 21-OHase mRNA was determined by RNA blot hybridization. The IIA1 clone reproducibly showed at least 10-fold increases in 21-OHase mRNA following ACTH treatment (Fig. 4*B*). The IC5 transfectant, which has higher basal levels of 21-OHase mRNA than IIA1, showed a smaller but significant increase in 21-OHase expression. These data thus strongly suggest that the transfected *21-OHase* gene retains sequences involved in ACTH transcriptional regulation.

DISCUSSION

Previous studies have defined two murine *21-OHase* genes encoded by the *S* region of the *H-2* complex (9). In this report, we utilize gene-specific oligonucleotide probes and DNA-mediated gene transfer to explore the expression of these genes. Our results demonstrate that *21-OHase A* is expressed in adrenal glands and transfected Y1 cells, whereas no expression of *21-OHase B* is seen. We cannot exclude the possibility that *21-OHase B* is expressed in other tissues; however, we have not detected any 21-OHase RNA in blots of kidneys, liver, testes, or ovaries (unpublished data). Further studies are needed to define whether the apparent nonexpression of *21-OHase B* results from a defect in the structural gene or from altered regulatory sequences. White *et al.* (10) have proposed that only the human *21-OHase B* gene is expressed, but were not able to define the molecular basis for nonexpression of the human *21-OHase A* gene. Transfection experiments using human *21-OHase* genes and Y1 cells should provide new information regarding expression of the human *21-OHase* genes. In addition, this methodology may provide new insights into the molecular mechanisms underlying human 21-OHase deficiency in patients with congenital adrenal hyperplasia.

The expression of *21-OHase A* by E-7 transfectants raises several interesting questions. Y1 cells in culture do not normally express *21-OHase*, despite the presence of the *21-OHase* genes on Southern blots (unpublished data). Why then is the transfected *21-OHase A* gene expressed in Y1 cells following DNA-mediated gene transfer? Perhaps the simplest explanation is that expression of the endogenous *21-OHase* is prevented by mutations in regulatory or structural elements of the *21-OHase* genes. Alternatively, the presence within the genome of extra copies of the *21-OHase A* gene may serve to saturate a soluble repressor molecule. This would presumably allow expression of both the endogenous and transfected genes. Finally, the detectable expression of 21-OHase mRNA in E-7 transfectants may merely reflect the presence of multiple copies of this gene. Further studies are required to examine these possibilities.

The transfected genes studied in this report display tissue-specific expression; transfection of E-7 confers *21-OHase* expression to Y1 adrenocortical tumor cells but not to L cells (unpublished data). Moreover, the *sex-limited protein* gene, which is normally expressed only in hepatocytes (25), is not expressed in Y1 cells. Further analysis of Y1 transfectants may allow the definition of factors involved in tissue specificity, such as tissue-specific enhancers or soluble regulatory factors.

Our results indicate that the expression of *21-OHase* in E-7 transfected Y1 cells is regulated by ACTH. It is perhaps not surprising that this is the case, since Y1 cells have been noted previously to regulate the synthesis of other steroidogenic enzymes such as 11β-hydroxylase and adrenodoxin (26, 27). In view of recent reports that demonstrate a role for cAMP in ACTH effects on adrenal cells, it will be very interesting to analyze the 21-OHase response to ACTH in a mutant Y1 line deficient in cAMP dependent protein kinase activity (28). The Y1 transfectants may thus provide an excellent model for further studies of the mechanism of regulation of adrenal steroidogenesis.

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1. Simpson, E. R. & Waterman, M. R. (1983) *Can. J. Biochem. Cell. Biol.* **61,** 692–707.
2. Kominami, S., Ochi, H., Kobayashi, Y. & Takemori, S. (1980) *J. Biol. Chem.* **255,** 3386–3394.
3. New, M. I., Dupont, B., Grumbach, K. & Levine, L. S. (1982) in *The Metabolic Basis of Inherited Disease*, eds. Stanbury, J. B., Wyngarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S. (McGraw-Hill, New York), pp. 973–1000.
4. Kramer, R. E., McCarthy, J. L., Simpson, E. R. & Waterman, M. R. (1983) *J. Steroid Biochem.* **18,** 715–723.
5. Funkenstein, B., McCarthy, J. L., Dus, K. M., Simpson, E. R. & Waterman, M. R. (1983) *J. Biol. Chem.* **258,** 9398–9405.
6. Kramer, R. E., Simpson, E. R. & Waterman, M. R. (1983) *J. Biol. Chem.* **258,** 3000–3005.
7. Kramer, R. E., Rainey, W. E., Funkenstein, B., Dee, A., Simpson, E. R. & Waterman, E. R. (1984) *J. Biol. Chem.* **259,** 707–713.
8. White, P. C., New, M. I., Dupont, B. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7505–7509.
9. White, P. C., Chaplin, D. D., Weis, J. H., Dupont, B., New, M. I. & Seidman, J. G. (1984) *Nature (London)* **312,** 465–467.
10. White, P. C., Grossberger, D., Onufer, B. J., Chaplin, D. D., New, M. I., Dupont, B. & Strominger, J. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1089–1093.
11. Yasumura, Y., Buonassisi, V. & Sato, G. H. (1966) *Cancer Res.* **26,** 529–535.
12. Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C. & Axel, R. (1977) *Cell* **11,** 223–232.
13. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1,** 327–341.
14. Blin, N. & Stafford, D. (1976) *Nucleic Acids Res.* **3,** 2303–2308.
15. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
16. Southern, E. (1975) *J. Mol. Biol.* **98,** 503–517.
17. Chaplin, D. D., Woods, D. E., Whitehead, A. S., Goldberger, G., Colten, H. R. & Seidman, J. G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6947–6951.
18. Whitehead, A. S., Bruns, G. A. P., Markham, A. F., Colten, H. R. & Woods, D. E. (1983) *Science* **221,** 69–71.
19. Vieira, J. & Messing, J. (1982) *Gene* **19,** 259–268.
20. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
21. Yuan, P.-M., Nakajin, S., Haniu, M., Shinoda, M., Hall, P. F. & Shively, J. E. (1983) *Biochemistry* **22,** 143–149.
22. Kowal, J. & Fiedler, R. (1968) *Arch. Biochem. Biophys.* **128,** 406–421.
23. Pierson, R. W., Jr. (1967) *Endocrinology* **81,** 693–707.
24. Schimmer, B. P. (1969) *J. Cell. Physiol.* **74,** 115–122.
25. Saunders, D. & Edidin, M. (1974) *J. Immunol.* **112,** 2210–2218.
26. Kowal, J. (1969) *Biochemistry* **8,** 1821–1831.
27. Assano, K. & Harding, B. W. (1976) *Endocrinology* **99,** 977–987.
28. Rae, P. A., Gutmann, N. S., Tsao, J. & Schimmer, B. P. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1896–1900.