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Genetics

Nucleotide sequence analysis of murine 21-hydroxylase genes: Mutations affecting gene expression

(steroid biosynthesis/major histocompatibility complex/pseudogene/cytochrome P-450/*CA21H* genes)

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ABSTRACT **Steroid 21-hydroxylase [21-OHase; steroid 21-monooxygenase; steroid, hydrogen-donor:oxygen oxidoreductase (21-hydroxylating); EC 1.14.99.10] is a cytochrome P-450 enzyme required for the adrenal synthesis of mineralocorticoids and glucocorticoids. The gene encoding this protein is present in two copies (*21-OHase A* and *B*) in the *S* region of the murine major histocompatibility complex. Previous studies utilizing gene-specific oligonucleotide probes and gene transfer showed that only the *21-OHase A* gene is expressed in the BALB/c mouse. Here, we present the complete primary structures of both BALB/c 21-OHase encoding genes. Comparison of the nucleotide sequences defines a deletion of 215 nucleotides spanning the second exon of the *21-OHase B* gene; other nucleotide changes in the *21-OHase B* gene introduce frame shifts and premature termination codons. Southern blot analysis of C57BL/6 and DBA/2J mice indicates that a similar deletion is present in these strains; however the C3H/HeJ strain is a structural variant. A hybrid gene composed of the *21-OHase B* promoter placed 5′ of the *21-OHase A* structural sequences was efficiently transcribed following transfection into Y1 adrenocortical tumor cells. These findings demonstrate that the *21-OHase B* gene promoter is functional and suggest that mutations within the *21-OHase B* structural gene are responsible for its lack of expression.**

Adrenal steroid biosynthesis proceeds through three parallel pathways that end in the production of mineralocorticoids, glucocorticoids, and androgenic hormones (for review, see ref. 1). The adrenal steroid 21-hydroxylase enzyme [21-OHase; steroid 21-monooxygenase; steroid, hydrogen-donor:oxygen oxidoreductase (21-hydroxylating); EC 1.14.99.10] is required for the production of both aldosterone, the predominant mineralocorticoid in humans, and cortisol, the primary glucocorticoid. 21-OHase is a member of the superfamily of cytochrome P-450 enzymes, heme-containing metalloproteins that function as terminal oxidases and require NADPH-dependent cytochrome reductases as coenzymes. The enzyme is membrane bound (2) and has a molecular weight of approximately 55,000 (3). In the adrenal gland, corticotropin (ACTH) regulates the expression of *21-OHase* (in the human *CA21H*) by stimulating its transcription, thus leading to increased steady state levels of the protein (4, 5). Primary structure studies utilizing amino acid sequences of protein purified from adrenal glands (3, 6) and nucleotide sequences of cDNAs derived from human and bovine adrenals (7–9) and of human genomic clones (8, 10) have defined a leucine-rich hydrophobic region at the amino terminus that may be involved in the interaction of the enzyme with the endoplasmic reticulum; the heme moiety is bound within the carboxyl-terminal portion of the molecule. Comparison of the primary structure of the adrenal 21-OHase enzyme with those of hepatic cytochrome P-450 enzymes reveals significant homology, especially in the amino-terminal portion of the molecules (9–11).

Deficiency of 21-OHase is common in humans, occurring in approximately 1 in 5000 live births and accounting for approximately 95% of cases of congenital adrenal hyperplasia (1). Linkage studies have determined that this deficiency maps within the *HLA* major histocompatibility complex (for review, see ref. 1). By using cDNA probes, it has been possible to characterize and map precisely both the human (12, 13) and murine (14, 15) *21-OHase* genes. These studies indicate that both human and murine 21-OHase encoding genes lie within a duplicated portion of the class III region of the MHC. In the BALB/c mouse, the duplicated *21-OHase* genes, termed *21-OHase A* and *B*, are several kilobases (kb) 3′ of the genes encoding the murine sex-limited protein variant of C4 (*Slp*) and fourth component of complement (*C4*), as indicated in Fig. 1. In humans, the 21-OHase encoding genes (*CA21HA* and *CA21HB*) are observed in corresponding positions adjacent to the duplicated *C4* genes (12, 13).

An initial characterization of the expression of the murine *21-OHase* genes was performed using DNA-mediated gene transfer of the BALB/c *21-OHase A* and *B* genes (5). Transfection of the cloned *21-OHase A* gene into the Y1 adrenocortical tumor cell line led to expression of 21-OHase mRNA and bioactivity. This expression was stimulated by ACTH. In contrast, no 21-OHase mRNA was detected in transfectants bearing the cloned *21-OHase B* gene. The *in vivo* expression of the *21-OHase A* and *B* genes was evaluated by hybridization of BALB/c adrenal RNA with gene-specific oligonucleotide probes. 21-OHase-specific transcripts were detected only by the oligonucleotide corresponding to the *21-OHase A* gene, suggesting that the BALB/c *21-OHase B* gene is not normally transcribed in adrenal glands.

In this report, we present a detailed analysis of the primary structures of the *21-OHase A* and *B* genes derived from the BALB/c mouse. These sequences confirm the striking sequence homology between the two genes, with 96% homology in the protein coding regions. Similar sequence conservation is seen in the 5′- and 3′-untranslated regions. However, the presence of a deletion spanning 215 bases, including the second exon of the *21-OHase B* gene, provides a possible explanation for its lack of expression. Further, the *21-OHase B* gene also contains nucleotide changes that result in frame shifts and premature termination codons. Southern blot experiments indicate that a similar deletion is present in several common inbred mouse strains. Finally, we demonstrate that a hybrid gene containing the *21-OHase B* promoter placed 5′ to the *21-OHase A* structural gene can direct the transcription of 21-OHase mRNA when transfected into Y1

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Abbreviations: 21-OHase, adrenal steroid 21-hydroxylase; kb, kilobase(s).

cells. These results suggest that the lack of expression of the *21-OHase B* gene results from mutations downstream of the promoter.

MATERIALS AND METHODS

Materials. Restriction and modification enzymes were obtained from New England Biolabs. Radionuclides were purchased from Amersham. Dideoxy- and deoxynucleotide triphosphates were from Pharmacia P-L Biochemicals or Amersham. S1 nuclease was from Boehringer Mannheim. Oligonucleotides were synthesized by the solid-phase phosphoramidite technique using an Applied Biosystems (Foster City, CA) 380A synthesizer. Media, serum supplements, antibiotics, and geneticin (G418) were from GIBCO. Mice (6–8 weeks old) were obtained from The Jackson Laboratory.

Nucleic Acid Analysis. DNA of high molecular weight and total cellular RNA were prepared and blot analyses (Southern and RNA) were done as previously described (16). S1 nuclease protection experiments were done according to the method of Taub *et al.* (17). Primer extension analyses were performed as described (18).

DNA Sequence Analysis. Restriction fragments of the cosmid clones E-7 and E-26 (16) were inserted into phage M13 vectors mp18 or mp19, and nucleotides were sequenced using the dideoxynucleotide chain-termination method (19). When suitable restriction enzyme sites were unavailable for sequencing using the universal phage M13 sequencing primer, specific oligonucleotide primers were synthesized permitting overlap with previously determined sequences of the M13 subclones.

Construction of 21-OHase Plasmids. All plasmid constructions were prepared using standard techniques (20) and were subcloned into pUC18 (21). The plasmid containing the hybrid gene, p21-OHB$_{prom}$/21-OHA$_{struc}$, was prepared by using a 0.7-kb *Kpn* I–*Bam*HI DNA fragment of the *21-OHase B* gene that contains sequences from 530 bases 5′ of the transcription initiation site through 182 bases of the first exon (Fig. 1). A 3.8-kb *Bam*HI fragment that contains the downstream portions of the *21-OHase A* structural gene as well as 1 kb of DNA 3′ of the polyadenylylation site was inserted in the proper orientation behind this fragment. Cosmid E-26 (16) contains the entire *21-OHase B* structural gene as well as approximately 15 kb of 5′ sequences and 10 kb of 3′ sequences.

Cell Culture and DNA-Mediated Gene Transfer. Culture of mouse Y1 cells and gene transfer were done as described (5).

RESULTS

Structures of the Murine 21-Hydroxylase Genes. The nucleotide sequences of the *21-OHase A* and *B* genes of the

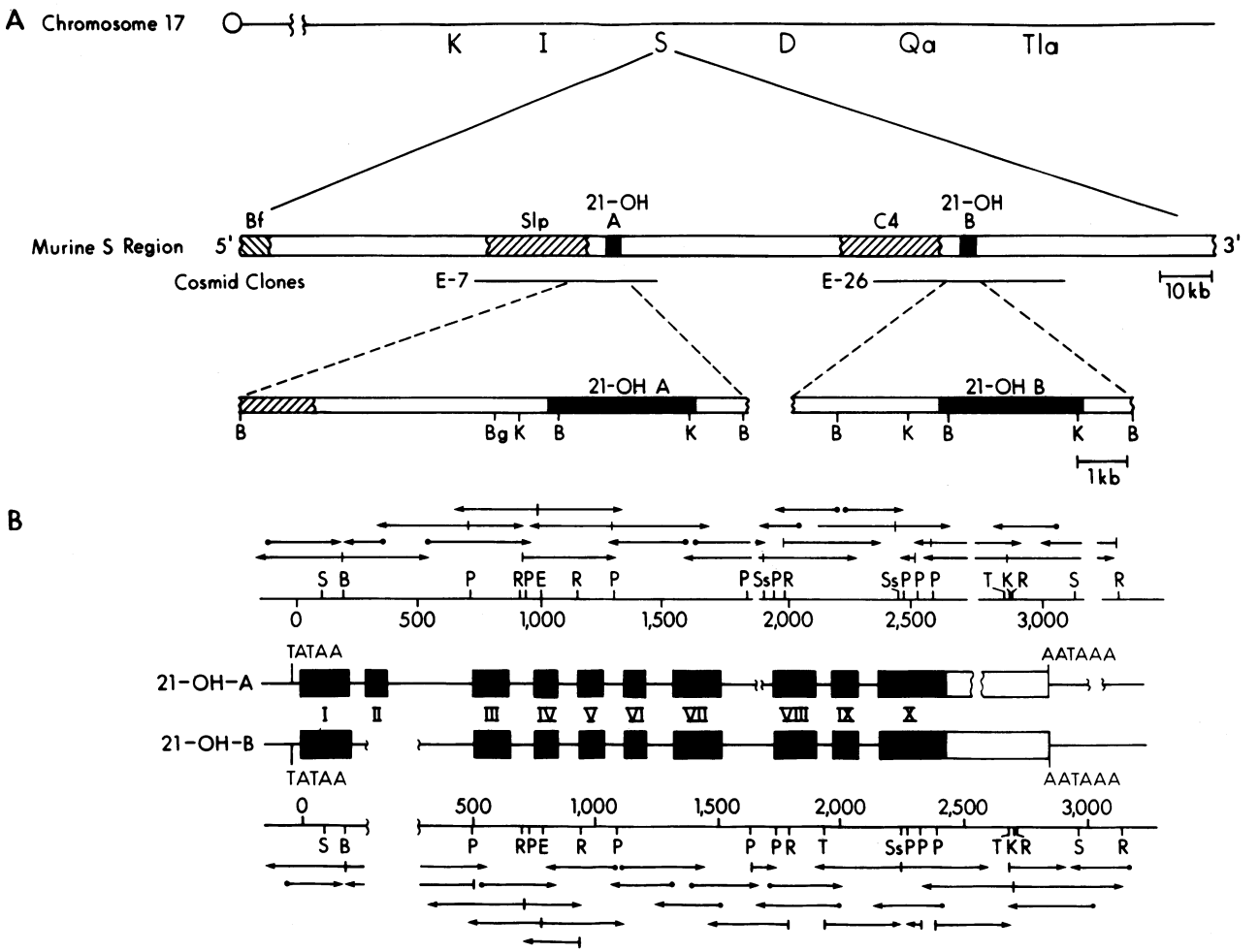

FIG. 1. Map of the *S* region of the murine major histocompatibility complex and the *21-OHase* genes. (*A*) The position of the *S* region within the MHC is as indicated. The positions of the class III MHC genes encoding factor B (*Bf*), sex-limited protein (*Slp*), the fourth component of complement (*C4*), and cosmid clones E-7 and E-26 are from ref. 16. (*B*) Exon structure and sequencing strategy for the *21-hydroxylase A* and *B* genes. Dideoxy chain-termination sequencing reactions using the universal sequencing primer are indicated by arrows originating from vertical bars. Reactions using specific primers derived from determined 21-OHase sequences are indicated by arrows originating from dots. Restriction enzyme cleavage sites are as indicated: B, *Bam*HI; E, *Eco*RI; K, *Kpn* I; P, *Pst* I; R, *Rsa* I; S, *Sma* I; Ss, *Sst* I; T, *Taq* I.

BALB/c mouse are shown in Fig. 2. Comparison of these sequences indicates that they share >90% homology over the full extent of the coding and noncoding sequences. The proper reading frame and exon/intron boundaries of the *21-OHase A* gene were determined by comparison of its sequence with previously published sequences of *21-OHase* (6–11, 22), and adhering to the G-T/A-G splice donor/acceptor site rule (23). In all cases, the location of exon boundaries was confirmed by using S1 nuclease protection analysis (data not shown).

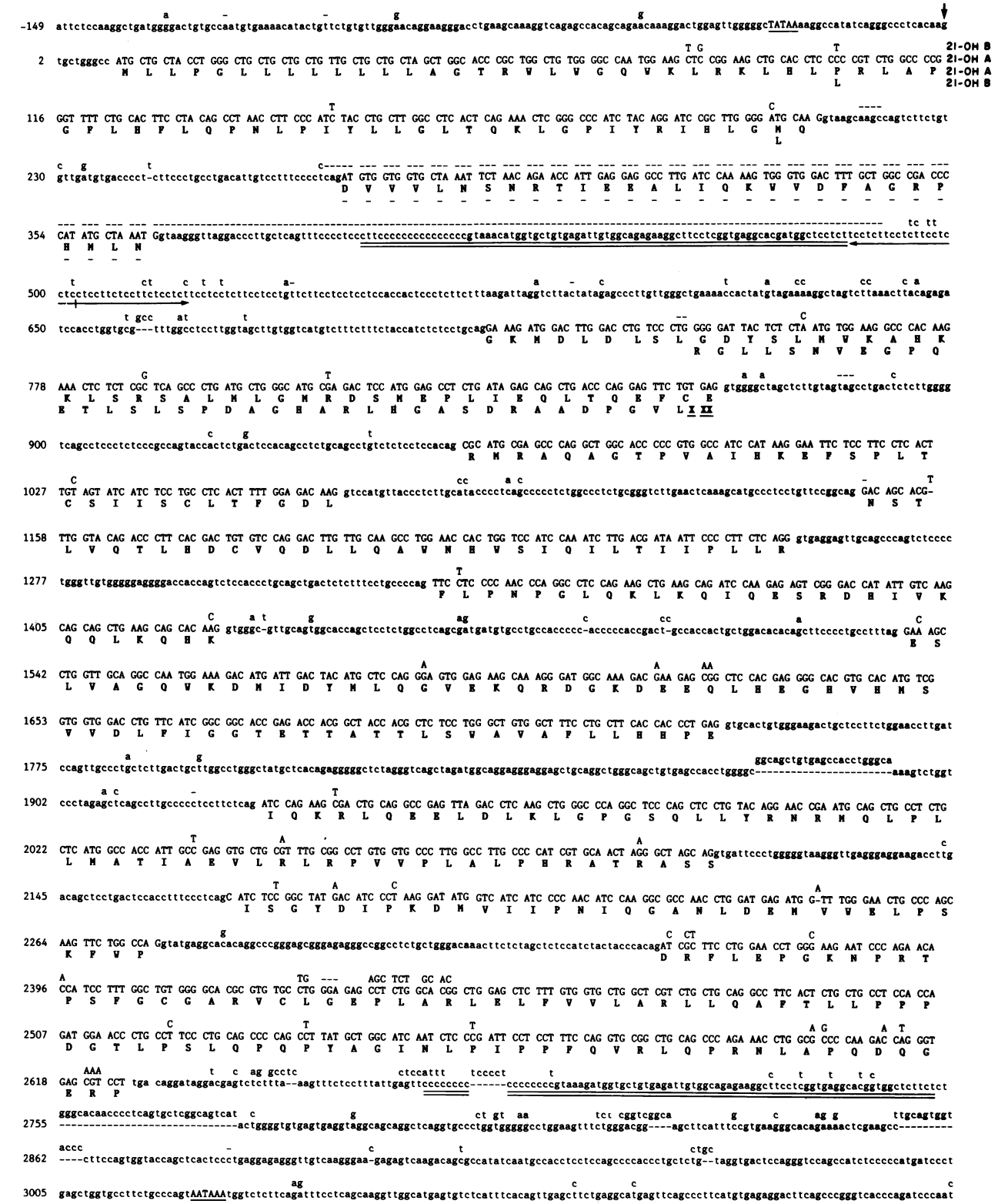

FIG. 2. Primary structures of the *21-OHase A* and *B* genes. The nucleotide sequence of the *21-OHase A* gene is indicated with base numbering starting at the major transcription initiation site indicated by a vertical arrow. The protein translation is indicated below the nucleotide sequence. The nucleotide sequence of the *21-OHase B* gene is shown above that for *21-OHase A*; blank spaces indicate identical sequences. Dashes indicate deleted bases. Differences in the protein translation of *21-OHase B* are indicated below that of *21-OHase A*. The protein translation of *21-OHase B* is not shown following the stop codon (XXX) at nucleotide position 858. The 81-base sequences that are duplicated at positions 401 and 2673 are indicated by double underlining. A 37-base sequence with dyad symmetry is indicated starting at position 483.

The *21-OHase A* gene encodes a protein of 487 amino acids and is divided into 10 exons. Both S1 nuclease protection and primer extension assays indicate that a 5′-untranslated region of only 10 nucleotides precedes the first exon (data not shown). The sequence "TATA", an element conserved in many eukaryotic promoters that is postulated to play an important role in determining proper initiation sites for RNA polymerase II (23), is located 29 nucleotides 5′ of the transcription initiation site. The first exon contains 23 leucines forming the core of a hydrophobic domain that may play a role in anchoring the enzyme to the microsomal membrane. Exon 10 contains the cysteine thought to be involved in binding of heme within the enzyme (24). A 3′ untranslated region of approximately 400 bases ends with the polyadenylylation signal AATAAA at position 3027. Of particular interest, the second intron contains an 81-base-long sequence rich in T+C that is repeated with >96% homology in the 3′-untranslated region (indicated by double underlining in Fig. 2). The first 81-base sequence is immediately followed by a 37-base sequence that demonstrates an internal dyad symmetry.

Comparison of the Structures of the *21-OHase A* and *B* Genes. The aligned sequences of the *21-OHase A* and *B* genes indicate infrequent single base differences within the 150 nucleotides 5′ to the transcription initiation site. Comparison of the sequences within the body of the structural genes shows that nucleotides corresponding to positions 274–488 of the *A* gene are deleted from the *B* gene. These sequences include the entire second exon. Interestingly, the 3′ boundary of the deleted region is located just 6 bases after the 81-base repeat, within the 37-base sequence described above. In addition to this deletion, the *21-OHase B* gene contains nucleotide changes or insertions as indicated in Fig. 2 that produce frame shifts and result in the introduction of premature termination codons.

Analysis of the *21-OHase B* Deletion in Different Mouse Strains. In order to assess whether the 215-base deletion observed in the BALB/c mouse is present in other mouse strains, genomic DNAs from BALB/c, C57BL/6, DBA/2J, and C3H/HeJ were analyzed by Southern blotting. When an 821-base *Bam*HI–*Eco*RI fragment of the *21-OHase A* gene is used as a probe of *Apa* I-restricted DNA, a 1.9-kb hybridizing band is observed in the *21-OHase A* gene (Fig. 3 *Left*, E-7), whereas a 1.7-kb hybridizing band is observed in the *21-OHase B* gene (Fig. 3 *Left*, E-26). Both of these bands are seen in Southern blots of *Apa* I-digested genomic DNA from BALB/c, C57BL/6, and DBA/2J mice (Fig. 3 *Right*) indicating the presence of a similar or identical deletion in these strains. *Apa* I-digested C3H/HeJ DNA contains a hybridizing band of 1.8 kb. An understanding of the differences in the *21-OHase* gene(s) in the C3H mouse will require a detailed analysis of the genes cloned from this strain.

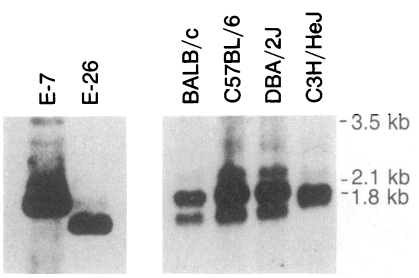

FIG. 3. Southern blot analysis of the deleted region of *21-OHase B* in different mouse strains. Cosmid and genomic DNA were digested to completion with *Apa* I, fractionated by electrophoresis in 1.2% agarose, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled *Bam*HI–*Eco*RI fragment spanning nucleotides 178–999 of the *21-OHase A* gene. After washing in 0.2 × standard saline citrate (30 mM NaCl, 3 mM sodium citrate) containing 0.1% $NaDodSO_4$ at 56°C, hybridizing bands were identified by autoradiography. (*Left*) Digests of the cosmids E-7 and E-26. (*Right*) Digests of genomic DNA from the indicated mouse strains. Sizes of hybridizing bands were estimated by comparison with the migration of fragments of lambda DNA digested with *Hin*dIII.

Analysis of Function of the 21-OHase B Promoter. One explanation for the lack of expression of the *21-OHase B* gene both *in vivo* and *in vitro* was that sequence changes in the 5′-flanking region resulted in loss of promoter activity. To test this hypothesis, we constructed a hybrid gene in which a 700-base *Kpn* I–*Bam*HI fragment containing the *21-OHase B* promoter and the first 182 nucleotides of its first exon was substituted for the corresponding region of the *21-OHase A* gene. This plasmid was then introduced into Y1 cells by DNA-mediated gene transfer, and the presence of *21-OHase*-specific transcripts was assessed by RNA blot analysis. The results (Fig. 4) demonstrate clearly that the *21-OHase B* promoter is functional; an RNA species of the same size as that present in adrenal RNA (Fig. 4, lane 5) is found in Y1 cells transfected with the hybrid gene (Fig. 4, lanes 1 and 2). In agreement with our previous report (5), no 21-OHase mRNA is detected in Y1 cells transfected with the unmodified *21-OHase B* gene (Fig. 4, lanes 3 and 4).

DISCUSSION

In this report, we present the complete nucleotide sequence of the *21-OHase A* and *B* genes in the BALB/c mouse. Comparison of the sequences shows a deletion in the *21-OHase B* gene of 215 bases including the second exon, as well as several single base changes that result in premature termination codons. These mutations provide an explanation for the finding that the *21-OHase B* gene is expressed neither in mouse adrenal glands nor when transfected into Y1 cells (5). The possibility that the inactivity of the *21-OHase B* gene was due to the scattered nucleotide changes within its promoter region was assessed by experiments using a hybrid gene containing the *21-OHase* B promoter placed 5′ of the structural portions of the *21-OHase A* gene. Y1 cells transfected with this hybrid gene contain apparently normal 21-OHase mRNA, indicating that the *21-OHase B* promoter is not intrinsically inactive. The deletion found in the *21-OHase B* gene of the BALB/c mouse was shown by Southern blot analysis to be a common feature of many inbred mouse strains suggesting that the lack of expression of the *21-OHase B* gene is not peculiar to the BALB/c mouse, and that *21-OHase B* is a pseudogene in most mouse strains.

Several features of the murine *21-OHase* gene are of interest. While the consensus promoter sequence TATAA is found at

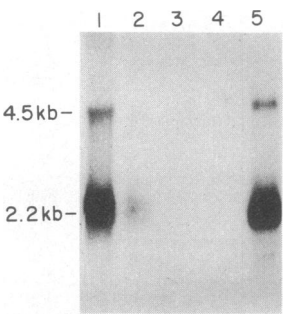

FIG. 4. Analysis of promoter activity of the *21-OHase B* gene. Total RNA was isolated from transfected clones (see *Materials and Methods*) and was analyzed by RNA blot hybridization by using as a probe a 1.6-kb *Bam*HI–*Sst* I fragment of *21-OHase A* labeled with ^{32}P by nick-translation. Washing and autoradiography were described in the legend of Fig. 3. Lanes 1 and 2, 15 μg of RNA from independent isolates transfected with $p21\text{-}OHB_{prom}/21\text{-}OHA_{struc}$; lanes 3 and 4, 15 μg of RNA from independent isolates of cells transfected with cosmid E-26; lane 5, 5 μg of BALB/c adrenal RNA.

position −29 in both the *21-OHase A* and *B* genes, none of the other upstream promoter elements thus far identified (for review, see ref. 25) is found in these genes. However, of particular interest, the sequence GTCAGAG, matching a proposed consensus sequence for cAMP-mediated regulation of gene expression (26), is found at position −68 in both genes. Recent studies have shown that cAMP mediates the effect of corticotropin (ACTH) on *21-OHase* gene expression (27). Further studies will be required to determine whether this region of the *21-OHase* promoter is, in fact, involved in regulating its response to cAMP. Finally, it is intriguing that the 3′ boundary of the region deleted in the *21-OHase B* gene falls within the 37-base sequence with dyad symmetry, just 6 bases 3′ of the end of the 81-base duplicated region. It is attractive to postulate that these sequences played a role in the generation of the deletion; however, the molecular details of such a deletion event are not readily apparent.

The data presented indicate that the *21-OHase B* gene maintains a functional promoter. However, by using RNA blot transfer, we are unable to detect transcripts from the *21-OHase B* gene either in adrenal tissue or in Y1 cells transfected with the *21-OHase B* gene. It is likely that the premature stop codon or the deletion of the second exon itself leads to a marked decrease in the stability of its transcript. This model has been proposed to explain the very low levels of β-globin mRNA in a patient with β^0 thalassemia due to a nonsense mutation at a position corresponding to amino acid 17 (28). Another possibility is that regulatory sequences within the structural portion of the gene are required for normal transcription, and that these sequences may be altered or removed by the deletion in *21-OHase B*. Finally, it is possible that there are elements located 5′ of the *Kpn* I site used to prepare the hybrid gene construction that suppress transcription of the *21-OHase B* gene. These could prevent the expression of the intact *21-OHase B* gene, but since they would not be contained on the *21-OHase B* fragment used to prepare the hybrid gene, they would not suppress its expression.

The *21-OHase* gene falls within a duplicated region in both man and mouse (12–15). The major histocompatibility complexes (MHCs) of humans and mice are remarkably similar, and it is possible that the duplication of the primordial *21-OHase* gene and subsequent inactivation of one gene occurred prior to speciation. However, recent sequence analysis of the human 21-OHase genes *CA21HA* and *CA21HB* (8, 10) has demonstrated the presence of an 8-base deletion within the third exon of the human 21-OHase A encoding gene, introducing a premature termination codon. Thus, in humans, the 21-OHase A gene *CA21HA* is a pseudogene, whereas in the mouse, the *21-OHase B* gene is inactive. In addition, the nature of the deletions that prevent expression is different in the murine and human genes. It is thus reasonable to conclude that the inactivations of one *21-OHase* gene in each species represent independent events. This suggests that there may be deleterious effects from the expression of two functional *21-OHase* genes. Alternatively, there may be a strong advantage from duplication of either the *C4* gene or other as yet unidentified genes in the region. In this case, mutation and inactivation of one *21-OHase* gene would carry no selective disadvantage. A more complete understanding of the role of *21-OHase* regulation in the adrenal steroidogenic pathway and of the genetics of this region of the chromosome will be required before these possibilities can be fully evaluated.

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