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Androgen induction of alcohol dehydrogenase in mouse kidney. Studies with a cDNA probe confirmed by nucleotide sequence analysis

(Recombinant DNA; liver DNA library; gene cloning; genetic regulation)

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SUMMARY

A cDNA clone for the β -chain of human alcohol dehydrogenase (ADH) was used to isolate several cross-hybridizing clones from a mouse liver cDNA library. Clones pADHm9 and a portion of pADHm12 were sequenced. pADHm9 coded for a sequence of 151 C-terminal amino acids and some untranslated sequences from the 3' end of its corresponding mRNA. This clone was identified as an *Adh-1* cDNA clone. Consistent with the known expression of *Adh-1*, this gene was expressed constitutively in liver, whereas the *Adh-3* gene product was found only in stomach, lung and reproductive tissues. Furthermore, the translated region of the cDNA shared 91% amino acid sequence homology with rat liver ADH. [³²P]pADHm9 was used as a hybridization probe to study the mechanism of androgen induction of kidney ADH activity. Induction of A/J female mice by androgen resulted in a dramatic increase in the steady-state level of *Adh-1* mRNA content which correlated with the level of enzyme induction. The size of the mRNA obtained from control or induced kidney and liver tissues was indistinguishable by Northern analysis. [³²P]pADHm9 was also used to probe restriction fragments of genomic DNA obtained from several inbred mouse strains. The hybridization patterns, considered with the genetic evidence, suggested that pADHm9 recognized sequences which may be present as only a single copy in the genome. No restriction fragment length polymorphisms were observed among the several inbred mouse strains examined.

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Abbreviations: aa, amino acid(s); ADH, alcohol dehydrogenase; *Adh*, gene coding for ADH; bp, base pair(s); cDNA, DNA

complementary to messenger RNA; DEAE, diethylaminoethyl; kb, kilobase(s) or 1000 bp; mRNA, messenger RNA; nt, nucleotide(s); ORF, open reading frame; PA, polyacrylamide; PVP, polyvinyl pyrrolidone; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na₃·citrate (pH 7.8).

INTRODUCTION

The first step in the major route for the metabolism of ethanol in mammalian liver is catalysed by ADH. In the mouse the *Adh-1* gene on chromosome 3 encodes the major ADH (A_2) expressed in liver (Holmes et al., 1981). The *Adh-1* gene is defined by allelic variants which affect the electrophoretic mobility of the enzyme. Kinetic analyses support a major role for this enzyme in ethanol metabolism (Algar et al., 1983). Allelic variation at a single regulatory locus results in differences among inbred mouse strains in the temporal development of liver ADH activity. Thus, inbred strains possessing high and low levels of liver ADH activity as adults do not differ in ADH activity until after 25 days of development. Alleles at this temporal locus (designated *Adh-1t*) determine the level of ADH protein in liver by modulating the rate of ADH synthesis (Balak et al., 1982). This regulatory gene is tissue specific in its expression and affects level of expression of *Adh-1* in liver but not kidney tissue. As another parameter of regulation, a wild stock of Danish mice has higher activity of kidney ADH than any of the inbred lines examined but has a low liver activity (Holmes et al., 1982; M.R.F., unpublished observations). In addition to this genetic control, *Adh-1* is androgen-inducible in kidney (Ohno et al., 1970).

Another ADH possessing divergent substrate specificity and tissue expression is encoded by the *Adh-3* gene which is tightly linked to *Adh-1* (Holmes et al., 1981b). A tightly linked, *cis*-acting, temporal locus (*Adh-3t*) controls the tissue-specific expression of this enzyme (Holmes et al., 1981a). All inbred strains express this gene product in stomach, lung and kidney, but, due to allelic variation at the *Adh-3t* locus, certain strains fail to express this gene product in reproductive tissues.

In this paper we describe the isolation of a partial cDNA clone which by DNA sequence analysis appeared to code for mouse ADH-1. This clone was used to determine the size of the *Adh-1* mRNA. In addition, we confirmed that androgen induction of kidney ADH enzyme activity was accompanied by an increase in the amount of a putative *Adh-1* mRNA. *Adh-1* clones described here will extend the formal genetic and biochemical analysis such that the mouse ADH system will also provide insight into an understanding of the regulation of mammalian genes when analysed at the molecular level.

MATERIALS AND METHODS

(a) Enzymes, isotopes and animals

Restriction endonucleases were purchased from New England Biolabs or Bethesda Research Laboratories and used according to the suppliers' instructions. Polynucleotide kinase was purchased from P-L Biochemicals. Guanidinium thiocyanate was from Fluka. Labeled isotopes were from New England Nuclear. High- M_r DNAs from inbred mice were obtained from Dr. Ben Taylor of the Jackson Laboratory. Testosterone pellets for implantation were a generous gift of Dr. Kenneth Paigen, Department of Genetics, University of California at Berkeley. Inbred mice were obtained from the Jackson Laboratory and bred at the University of South Carolina.

(b) Selection of pADHm9

A cDNA library (Norgard et al., 1980) prepared from SWR/J mouse liver poly(A)⁺ RNA was provided by Dr. John J. Monahan, Roche Institute of Molecular Biology. Screening was done using standard protocols (Maniatis et al., 1982; Gergen et al., 1979; Grunstein and Hogness, 1975) using cDNA for the β -subunit of human ADH (Duester et al., 1984) as probe. Human ADH cDNA was purified from the recombinant plasmid by cleavage with *Pst*I, separation by agarose gel electrophoresis and subsequent recovery from DEAE membranes. Purified cDNA was labeled with [α ³²P]dCTP to a specific activity of $1-5 \times 10^8$ cpm/ μ g by nick-translation (Rigby et al., 1977). Hybridization was done in 3 ml of 50% formamide, 5 \times SSC, 100 μ g/ml salmon sperm DNA, 5 \times Denhardt (1966) medium, 150 μ g/ml yeast tRNA, 50 mM sodium phosphate, pH 6.5, and 100 μ g/ml poly(A) containing 3×10^6 cpm at 42°C for 18 h. The filters were washed in a solution of 5 \times SSC, 1% SDS, 0.1% PVP, 0.1% Ficoll, 5 mg/ml glycine for 2 h at 65°C and then twice in 2 \times SSC for 30 min at 65°C. Positive colonies were identified by autoradiography, picked and rescreened.

To increase the likelihood of detecting mouse cDNA clones containing coding sequences, a 300-bp *Pst*I-*Sau*3A fragment of the human cDNA was isolated by PA gel electrophoresis (Maxam and Gilbert,

1977) and labeled by replacement synthesis (Maniatis et al., 1982) to a specific activity of approx. 10^9 cpm/ μ g. In this fragment, 273 of the 300 bp represent sequences that are translated in human ADH mRNA.

(c) Restriction mapping and sequencing

Recombinant plasmids were isolated from positive colonies, and the size of their inserts was determined by agarose gel electrophoresis (Maniatis et al., 1982). Southern (1977) blots containing inserts from mouse cDNA clones were hybridized with nick-translated human ADH cDNA (Rigby et al., 1977) to confirm that mouse inserts were complementary to the human ADH cDNA. Restriction maps of several clones were obtained by standard means using single and double enzymatic digestions. One clone, designated pADHm9, was completely sequenced (Maxam and Gilbert, 1977). A portion of a second clone, pADHm12, that extends slightly further in the 5' direction, was also sequenced.

(d) RNA isolation and analysis

Total RNA was isolated using the guanidinium thiocyanate method (Chirgwin et al., 1979). Electrophoresis of RNA was done in formaldehyde-denaturing agarose gels (Lehrach et al., 1977) using 20 mM Hepes, pH 7.8, as the buffer. RNA was blotted onto nitrocellulose (Thomas, 1980) and hybridized (Andrews et al., 1982) to nick-translated [32 P]pADHm9 DNA.

RESULTS

(a) Identification and sequencing of a putative mouse ADH cDNA clone

About 7000 colonies were screened to identify cells harboring recombinant plasmids containing putative mouse liver ADH cDNA sequences using the 300-bp *Pst*I-*Sau*3A fragment of human ADH cDNA (Duester et al., 1984). This fragment primarily contains ADH-coding sequences thus increasing the probability that we selected plasmids coding for mouse ADH. After three sequential

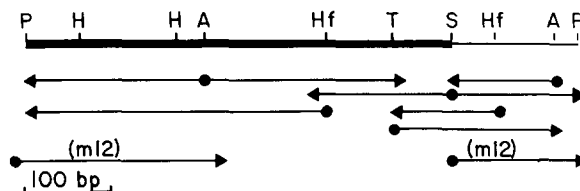


Fig. 1. Restriction map and sequencing strategy of pADHm9. Restriction sites were determined by single and double endonucleolytic digestions. Fragments were 5'-end labeled and sequenced in the directions indicated. Both strands and all overlaps were sequenced. Based upon the sequence analysis, the translated and untranslated 3'-end of the mRNA were shown in heavy and thin lines, respectively. Portions of pADHm12 (designated m12) were used for sequencing the 5'- and 3'-ends since this clone extends slightly further in both directions. P, *Pst*I; H, *Hae*III; A, *Alu*I; Hf, *Hin*II; T, *Taq*I; S, *Sau*3A.

rounds of colony hybridization, five clones were chosen for further study. Restriction mapping indicated that the inserts in these chosen recombinant plasmids were related and overlapped. The restriction map obtained for one plasmid, pADHm9, is shown in Fig. 1. The sequencing strategy shown below the map was followed and both strands including all overlaps were sequenced. Part of the strategy included partial sequencing of the cDNA insert in pADHm12, a nearly identical recombinant plasmid whose insert extended about 10 bp in either direction beyond the ends of the pADHm9 insert. The complete nt sequence was shown in Fig. 2. This sequence included a single ORF which encoded 151 C-terminal aa of the mouse ADH-1 protein and 133 bp of 3'-untranslated region. pADHm9 apparently did not contain the 3' end of the mouse ADH mRNA. The probable identity of this clone was based upon the homology of its encoded aa sequence to the aa sequence for rat ADH (Branden et al., 1975). A comparison of the rat and mouse ADH aa sequences was given in Table I.

(b) Androgen induction of RNA complementary to pADHm9

We next studied the ability of androgen to alter the steady-state levels of ADH enzyme activity and RNA sequences complementary to pADHm9 in mouse liver and kidney. Female A/J mice were implanted with androgen pellets and killed after 12 days. ADH enzyme activity in liver and kidney was determined for some animals while other ani-

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      223                230
      Asp Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Leu
1   G34 TG GAC ATC AAC AAG GAC AAG TTT GCC AAG GCC AAA GAC TTG 75
      240                250
      Gly Ala Thr Glu Cys Ile Asn Pro Gln Asp Tyr Ser Lys Pro Ile
76  GGT GCA AGT GAG TGC ATC AAC GCT CAA GAC TAC AGC AAA GCG ATC 120
      260                260
      Gln Glu Val Leu Gln Glu Met Thr Asp Gly Gly Val Asp Phe Ser
121 CAG GAA GTT CTC CAG GAG ATG ACC GAC GCA GGG GTG GAC TTT TCG 165
      270                280
      Phe Glu Val Ile Gly Arg Leu Asp Thr Met Thr Ser Ala Leu Leu
166 TTT GAA GTC ATC GGC GCG CTT GAG ACC ATG ACT TCT GCC CCG CTG 210
      290
211 Ser Cys His Ala Ala Cys Gly Val Ser Val Val Val Val Gly Val Pro
AGC TGC CAT GCA GCA TGT GGT GTA ACC GTC CTC CTA GGA CTC CCT 255
      300                310
      Pro Asn Ala Gln Asn Leu Ser Met Asn Pro Met Leu Leu Leu Leu
256 GCG AAT GCC CAG AAC ACC CTC TCC ATG AAC GCC ATG TTG CTG CTG CTG 300
      320
301 Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly Gly Phe Lys Ser Lys
GGA CGC ACC TGG AAG GGA GCA ATA TTT GGC GGG TTT AAC AGT AAA 345
      330                340
      Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met Ala Lys Lys Phe
346 GAT TCT GTC CCT AAA CTT GTG GCT GAC TTC ATG GCT AAG AAG TTT 390
      350
391 Pro Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe Glu Lys Ile
CGC TTC GAC GCG TTA ATF ACC CAT CTT TTA CCT TTC GAG AAA ATA 435
      360                370
      Asn Glu Ala Phe Asp Leu Leu Arg Ser Gly Lys Ser Ile Arg Thr
436 AAT GAA GCA TTT GAC CTG CTT CDT TCT GGA AAG AGC ATC CGT ACC 480
      374
      Val Leu Thr Phe End
481 GTC CTG ACT TTC TGA GATCATGTGG ATGCCTTCCC AGCCACCAAT 525
526 TTCTGAACCC TAACCCAGAC TGATTCAAAGC ACCAGCCACA TCACAGCCTT 575
576 AATCTTTGCT GTTTAGACAC ACACCCAAATA AACTACTGTI CTAAGCTCTC 625
626 CAT C19 647
    
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Fig. 2. Nucleotide sequence of a partial cDNA for mouse ADH. The aa sequence encoded in the ORF is indicated below the codons and corresponds to aa residues 223–374 in rat ADH (Brandon et al., 1975). A polyadenylation signal (AATAAA) occurred at nt 602–607 and is underlined.

mals in the same group were used for extraction of kidney and liver RNA. After 12 days of continuous testosterone administration, the increase in the specific activity of kidney ADH was nearly 15-fold (Table II). Semi-quantitation of the pADHm9-like sequences in RNA preparations from control and induced animals was determined by hybridization with nick-translated pADHm9 and autoradiography. As can be seen in Fig. 3, the RNA prepared

TABLE I

Amino acid substitutions between mouse liver and rat liver ADH^a

Residue	Mouse ADH	Rat ADH	Change in property
239	glu	asp	
247	ser	thr	
255	gln	thr	
258	thr	ser	
276	thr	ala	nonpolar to polar
284	ala	ser	polar to nonpolar
291	val	ile	
297	asn	val	nonpolar to polar
300	asn	ser	
303	met	val	
327	ser	ala	nonpolar to polar
343	asp	glu	

^a Total residues available for comparison are from aa 223 to 374. The change in property is from the rat to the mouse ADH.

from kidney tissue of induced animals contained a greater abundance of sequences complementary to pADHm9 than uninduced animals. Based upon densitometry of serially diluted RNA samples (Fig. 3B), the induction pADHm9-like RNA sequences in androgen-treated animals was estimated to be 15- to 20-fold greater than in control animals. This correlated with the increase in specific activity of kidney ADH after 12 days of androgen induction. As can be seen in Fig. 3A, there was no detectable effect of androgen administration upon RNA levels complementary to pADHm9 in liver tissue.

Total cellular RNA was isolated and separated by electrophoresis on denaturing agarose gels. Putative ADH mRNA was located on Northern blots by hybridization to nick-translated pADHm9 (Fig. 4).

TABLE II

Androgen induction of ADH in kidney and liver tissues of A/J female mice

Results are mean ± standard deviation for at least three animals in each group. Mice were androgen-induced for 12 days with implanted testosterone pellets. ADH activity was determined as previously described (Balak et al., 1982). Protein content in tissue homogenates was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Treatment	ADH activity		
	Kidney (specific activity)	Kidney (units/g)	Liver (units/g)
Control	0.012 ± 0.001	0.71 ± 0.06	27.4 ± 1.9
Androgen-induced	0.158 ± 0.055	10.5 ± 0.75	24.5 ± 3.6

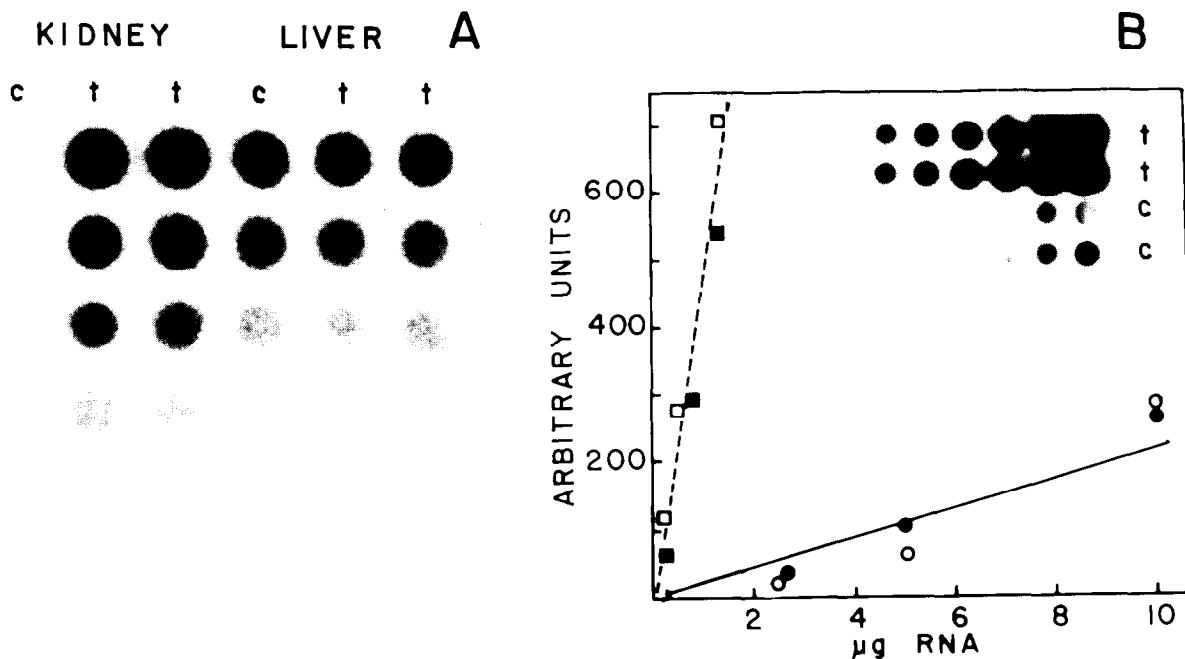


Fig. 3. Dot blot analysis of the androgen induction of kidney ADH mRNA. Approx. 10, 5, 2.5, 1.25, 0.625 and 0.312 mg of total cellular RNA (top to bottom in panel A, right to left in panel B) were loaded onto nitrocellulose membranes and hybridized with 5×10^6 cpm of nick-translated [32 P]pADHm9 whole plasmid (panel A) or [32 P]cDNA (3×10^6 cpm) insert isolated from pADHm9 (panel B); c, control; t, testosterone-treated. In panel B, two control and two testosterone-treated kidney RNA samples were used, and the autoradiographs were scanned and integrated with a densitometer. \circ , \bullet , control; \square , \blacksquare , androgen-induced. Open and closed symbols represent duplicate samples.

Administration of androgen clearly increased the relative concentration of a discrete 1.5-kb RNA in kidney that was complementary to pADHm9. Both induced and control kidney RNAs appeared as single electrophoretic species that was not distinguishable by size from a corresponding liver RNA.

(c) Analysis of pADHm9 sequences in genomic DNA

Genomic Southern blots were prepared using high- M_r DNAs obtained from seven inbred strains of mice that were digested with two restriction enzymes which did not recognize sequences in pADHm9 cDNA. Radiolabeled pADHm9 hybridized to 4.1- and 1.1-kb *Eco*RI fragments and 5.2- and 3.7-kb *Bam*HI fragments (Fig. 5). The same pattern of hybridizing fragments was observed in DNA from all seven inbred strains.

DISCUSSION

Three lines of evidence strongly supported the idea that pADHm9 was an *Adh-1* cDNA clone. (1) *Adh-1* (but not *Adh-3*) is expressed in liver and codes for an enzyme with kinetic properties like the class I human ADH (Algar et al., 1983), a group which includes the β subunit of ADH (Strydom and Vallee, 1982). An ADH which uses longer-chain alcohols is found in mouse liver but is not immunologically cross reactive with the *Adh-1* gene product (Algar et al., 1983). Therefore, a clone identified in a mouse liver cDNA library by cross-hybridization with a human β -chain ADH cDNA probe would most likely be an *Adh-1* cDNA clone. (2) The aa sequence for the 151 C-terminal residues encoded in the cDNA sequence had a 91% homology with the aa sequence of rat liver ADH (Branden et al., 1975). Furthermore, the 12 aa substitutions occurring in a comparison between mouse and rat sequences are quite conservative and only 4 aa involve a change in property of the aa at the variant position in the mouse

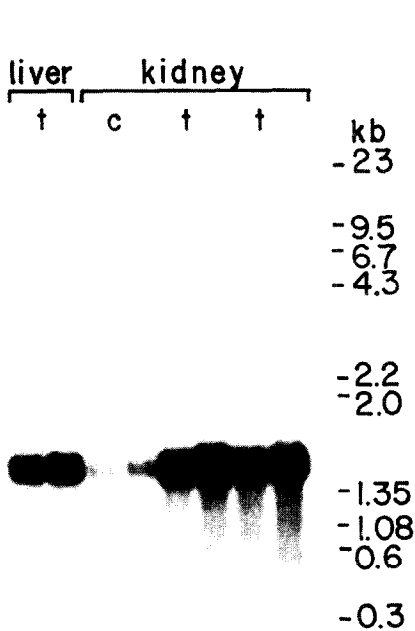


Fig. 4. Electrophoresis of kidney RNA from control and androgen-induced A/J mice. A Northern blot was made from a denaturing agarose gel that contained the samples indicated above the gel and hybridized with nick-translated insert from pADHm9. Each sample was loaded in duplicate with 5 μ g and 10 μ g of RNA being used in the left- and right-hand lanes, respectively. Positions of M_r markers (λ HindIII and ϕ X174 HaeIII DNA fragments) were indicated on the right margin. c, control; t, testosterone treated for 12 days.

protein. (3) The finding that pADHm9 mRNA was androgen-inducible in kidney was consistent with the known increase in kidney ADH activity after treatment with testosterone (Ohno et al., 1970). Finally, the observed increase in enzyme-specific activity after implantation of testosterone pellets correlated well with the observed increase in the steady-state level of a 1.5-kb androgen-inducible RNA species measured by hybridization with pADHm9. Therefore, we concluded that the cDNA insert within pADHm9 was derived from mouse *Adh-1* mRNA.

Additional proteins in mouse kidney are androgen-inducible other than ADH, and the mechanisms have been studied in some detail. For instance, it is known that the induction of ornithine decarboxylase is accompanied by an increase in the specific protein as measured immunologically (Seely and Pegg, 1983) and by an increase in the corresponding mRNA

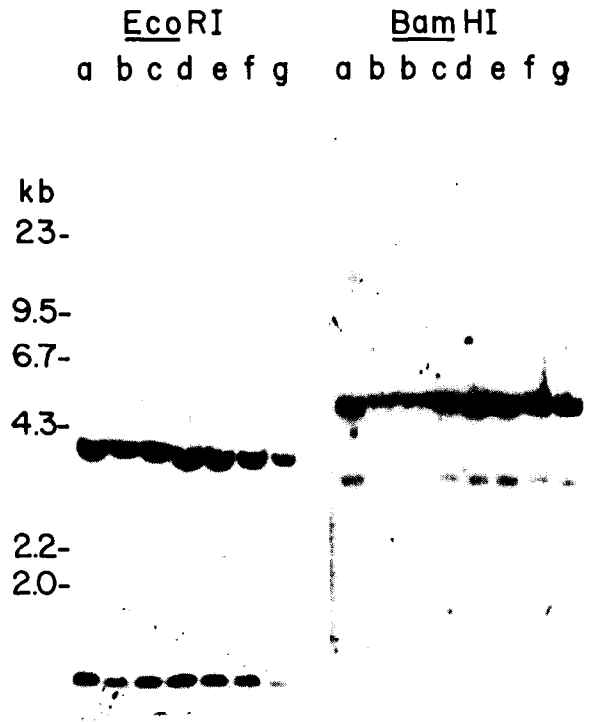


Fig. 5. Sequences in genomic DNA which hybridize to pADHm9. About 20 μ g of high M_r weight DNA from seven inbred mouse strains were digested to completion with *Eco*RI or *Bam*HI and electrophoresed on 0.7% agarose gels. After transfer to nitrocellulose the fragments were identified by hybridization to a 32 P-labeled cDNA insert from pADHm9 and autoradiography for 3 days at -70° C using intensifying screens. The lanes contained digested DNA from the following inbred strains: (a) DBA/2J, (b) C3HeB/FeJ, (c) A/J, (d) Balb/cJ, (e) C57BL/6J, (f) AKR/J and (g) SWR/J. Positions of the λ HindIII markers (kb) are given on the left margin.

(Berger et al., 1984). Glucuronidase protein level and synthetic rates (Swank et al., 1973), and glucuronidase mRNA activity and concentration (Paigen et al., 1979; Watson et al., 1982; 1985; Catterall and Leary, 1983; Palmer et al., 1983) all increase after androgen stimulation. The androgen induction of alcohol dehydrogenase, like glucuronidase and ornithine decarboxylase, occurs by an increase in concentration of a specific mRNA for the protein. In all these cases, it is not yet known if this increase is due to a stimulation of transcription rates of the specific gene sequences or to mRNA stabilization.

Southern blot analysis of pADHm9 sequences in genomic DNA suggested that a single *Adh-1* gene exists. Alternatively, *Adh-1* may exist as multiple copies, each with the same restriction pattern. The

latter idea is argued against by the fact that allelic differences in the *Adh-1* gene exist which result in electrophoretic mobility differences in the ADH molecule (Holmes et al., 1981b). It seems unlikely that this single electrophoretic variant encoded in the variant *Adh-1^b* allele would quickly become fixed in multiple copies of the *Adh-1* gene. However, a final conclusion about this will come from a detailed molecular analysis of genomic clones which are complementary to pADHm9 cDNA.

The availability of this cDNA clone for the mouse *Adh-1* gene should facilitate molecular analysis of the identified and biochemically characterized genetic variants which exert a regulatory effect over *Adh-1* expression. This will enable questions to be asked about the nature of the variants and whether they exert their control by modulating rates of *Adh-1* mRNA transcription or stability and whether alternate sites of control are affected.

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