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Molecular genetic studies on alcohol and aldehyde dehydrogenase: individual variation, gene mapping and analysis of regulation

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Introduction

Through analysis of alcohol and aldehyde dehydrogenase enzymes at the protein level, the number of gene loci which encode these enzymes has been determined (Smith *et al.*, 1972; Li & Mages, 1975; Pares & Vallee, 1981). The tissue distribution, and kinetic properties have been defined and in addition a number of allelic variants were identified (Smith *et al.*, 1973; Bosron *et al.*, 1980, 1983; Yoshida *et al.*, 1983). Refinement of purification procedures led to isolation of enzyme polypeptides suitable for amino-acid sequence analysis (Impraim *et al.*, 1982; Hempel *et al.*, 1984a,b,c; Buhler *et al.*, 1984). Availability of amino-sequence data led to the development of oligonucleotide probes to screen liver complementary (c) DNA libraries to isolate cDNA clones for alcohol and aldehyde dehydrogenases (Duester *et al.*, 1984; Smith, 1986; von Bahr-Lindstrom *et al.*, 1986). In certain instances, cDNA clones were isolated using antibodies to purified enzymes and expression libraries of liver cDNA (Ikuta *et al.*, 1986; Hsu *et al.*, 1985). Subsequently, cDNA clones were used to isolate genomic DNA clones and to examine the organization of the alcohol metabolizing genes. This paper will describe studies which we and our collaborators have carried out over the past 3 years primarily on class I alcohol dehydrogenase (ADH) and, to a lesser extent, on mitochondrial aldehyde dehydrogenase. In the case of ADH, the genomic probes which we have isolated for α -, β - and γ -ADH genes and the application of these probes to physical gene mapping, investigations of inherited variations and genetic linkage analysis will be described. Results of studies in human hepatomas indicate that definition of the gene mapping relationships may contribute to our understanding of the mechanism of their origin and to our understanding of the clinical manifestations in hepatomas. Analysis of RNA species in different tissues and detailed analysis of cDNA clones has led to insights into some of the factors which may be involved in the regulation of expression of class I alcohol dehydrogenases and mitochondrial aldehyde dehydrogenases (ALDH).

Physical chromosome mapping of the class I and class II ADH loci

Physical mapping of the ADH loci was initially accomplished using a β -ADH cDNA clone, pADH12 and a series of man-rodent hybrids. Since the three class I ADH genes (α , β and γ), have a high degree of sequence homology (Duester *et al.*, 1984; Ikuta *et al.*, 1986, von Bahr-Lindstrom

et al., 1986), under conditions of low and medium stringency the β -ADH cDNA probe hybridizes to restriction fragments from all three genes. On analysing Southern blots of genomic DNA of human, rodent and hybrid cell lines, we observed that all of the pADH12 hybridizing fragments characteristic of human DNA were present in hybrids containing human chromosome 4 (Smith *et al.*, 1984). Analysis of DNA from hybrids containing fragments of human chromosome 4, provided evidence that the class I ADH genes were located in the region 4q21-4q24 (Smith *et al.*, 1985). Confirmation of this physical assignment has been obtained in studies on the linkage relationships of ADH and other genes assigned to human chromosome 4 (see below).

Derivation of ADH genomic clones

The first series of ADH genomic clones were derived by screening of the Maniatis library of human genomic DNA in λ phage with the β -ADH cDNA clone pADH12. A second series of clones was derived by screening of either the Maniatis library or of a genomic DNA library prepared from a hybrid which contained chromosome 4 as the only human chromosome. In this second screening the most 5' region of the longest β -ADH genomic clone was used. The genomic clones were classified as being derived from the α -, β - or γ -ADH loci based on stringency of hybridization and sequence analysis in regions where amino-acid analysis had indicated that substitutions specific for α -, β -, or γ -ADH occurred (Duester *et al.*, 1986).

Based on the sizes of the genomic clones which were isolated and on the fact that no overlaps were detected in the restriction maps of the clones, it seems likely that the ADH genes are not immediately adjacent to each other (Duester *et al.*, 1986).

Detailed sequence analysis in the 5' region of β -ADH and its implications for studies on ADH regulation

Detailed sequence analysis in the 5' ADH region by Duester *et al.* (1986) led to the identification of a number of interesting features. Located 27 base pairs (bp) and 158 bp, respectively, upstream of the transcription initiation site, the β -ADH gene contains sequences which match closely to the TATA box and CCAT box. These sequences are associated with eukaryotic promoters (Breathnach & Chambon, 1981; Benoist *et al.*, 1980). At -250 bases there is a sequence which matches in seven out of eight bases with the enhancer core sequence and which is embedded in a purine-rich stretch of DNA. Enhancer elements are sequences of DNA which have been shown to influence gene transcriptional activity through enhancement of RNA polymerase binding (Weiher *et al.*, 1983). A number of investigators have determined that purine and pyrimidine asymmetry usually occurs in the region of enhancer sequences, and that this asymmetry is necessary for correct function of the enhancer elements (Gillies *et al.*, 1984). In a number of other gene systems investigators have succeeded in identifying DNA-binding

Abbreviations used: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; RFLP, restriction fragment length polymorphism; EGF, epidermal growth factor; IL 2, interleukin 2; lod, logarithm of the odds in favour of linkage.

proteins which bind to enhancer regions and modulate gene expression (Weinberger *et al.*, 1987).

Through detailed sequence analysis in the 5' region of β -ADH, Duester *et al.* (1986) also located two tandem sequences with a high degree of homology to elements which have been previously identified in other genes as being glucocorticoid responsive elements, i.e. DNA elements which bind the glucocorticoid receptor-hormone complexes (Scheiderer *et al.*, 1983).

Potential application of DNA sequence information to clinical identification of individuals with unusual forms of ADH or ALDH

DNA sequence information is now available for β -ADH and for mitochondrial ALDH. Furthermore, the nucleotide triplets which code for β -1 and β -2 ADH alleles (ADH2-1 and ADH2-2), and the nucleotides which code for ADH γ -1 and γ -2 alleles (ADH3-1, ADH3-2), have been characterized (Duester *et al.*, 1986; Ikuta *et al.*, 1986; von Bahr Lindstrom *et al.*, 1986), as have the nucleotides which give rise to ALDH1-1 and the ALDH1-2 alleles (Impraim *et al.*, 1982). It will therefore be possible to utilize the polymerase chain reaction (Saiki *et al.*, 1985; Mullis *et al.*, 1986) and DNA isolated from white blood cells to define these specific ADH and ALDH genotypes.

For application of this technique it is essential that the DNA sequence in the region of the mutation be known, as well as the nucleotide sequence in the regions flanking the mutation. Two oligonucleotides corresponding to regions which flank the mutation at opposite ends, are used as primers. These oligonucleotides act as primers for chain synthesis in the presence of DNA polymerase and nucleotides. Through successive cycles of DNA synthesis, the specific region of DNA sequence between the outermost oligonucleotides can be greatly amplified. Allele specific oligonucleotides can then be used to determine the presence of base mismatches at a specific location (Mullis *et al.*, 1986). Use of allele-specific oligonucleotides and the polymerase chain reaction has greatly enhanced diagnostic capabilities for a number of common inherited diseases including sickle cell anaemia (Saiki *et al.*, 1985).

Detection of restriction endonuclease polymorphisms in human α -, β - and γ -ADH genes

In our initial studies we identified regions of the α -, β - and γ -ADH genomic clones which were free of repetitive DNA sequences and which could be used to screen human leucocyte DNA for restriction fragment length polymorphisms (RFLPs) (Smith, 1986). ADH gene regions which were free of repetitive sequences and which detected RFLPs, were excised from the λ clones and inserted into PUC plasmid vectors. More recently, we have used the method of probe prehybridization with total human DNA to block repetitive

sequences which may be present in genomic clones. In this way we recently identified a 2.2 kb *Bam*H1 *Hind*III fragment in the 5' region of an α -ADH genomic clone which detects a *Msp*I polymorphism. Since the three class I ADH genes are closely homologous in their coding and non-coding regions (Ikuta *et al.*, 1986), it has been difficult to derive probes which are locus specific and hybridize to a single ADH gene. To determine the gene of origin of a particular ADH restriction fragment, it is necessary to vary the stringency of the post-hybridization washes and if possible, to have probes corresponding to all three genes in a particular gene region. Thus far six different RFLPs of the ADH genes have been identified. We have analysed three of the RFLPs in both Caucasian and Oriental populations and significant differences in allele frequencies have been found.

In Table 1 are summarized the ADH probes used for RFLP analysis, the specific restriction enzymes which detect DNA polymorphism, the DNA fragment sizes, and the allele frequencies.

Linkage relationships of ADH and other loci on human chromosome 4q

The ADH3 locus (γ -ADH) has thus far been most extensively used in genetic linkage analysis, primarily because of the fact that in Caucasians the pADH74 and pADH73 probes, both derived from the γ -ADH gene, detect polymorphisms with favourable informational content. Murray *et al.* (1987), determined the linkage relationships of ADH3 and ten other gene loci which have been mapped to human chromosome 4.

It is of interest to note that the genes for a number of liver-specific proteins map on chromosome 4q. These are the genes for Gc, vitamin D binding protein, the genes for albumin and α -fetoprotein and the genes encoding α -, β - and γ -fibrinogen (Kidd & Gusella, 1985). Two growth factor genes are also located on chromosome 4q: these are the genes for epidermal growth factor (EGF) and the gene for interleukin 2 (IL-2) (Francke *et al.*, 1986). EGF is considered to be one of the most important factors responsible for liver regeneration (Rubin *et al.*, 1982).

Linkage analysis by Murray *et al.* (1987), indicates that ADH3 maps closely to the genes for EGF and IL 2. In the case of the ADH IL 2 linkage the maximum logarithm of the odds in favour of linkage (lod) score is 3.78 at $\theta = 0$. For the ADH-EGF pair the maximum lod score is 13.04 at $\theta = 0.04$. Significant lod scores were also observed in analysis of linkage between ADH and albumin (ALB) at a greater distance: for ADH-ALB the maximum lod score was 5.33 at $\theta = 0.21$.

Analysis of ADH and linked markers in hepatomas

We have examined ADH enzymes in 20 hepatomas using starch gel electrophoresis. In 5 out of 20 tumours there was reversion to a predominantly fetal pattern of isoenzymes. In

Table 1. Frequencies of *Xba*I, *Rsa*I and *Msp*I polymorphisms in random Caucasian and Oriental individuals

Abbreviations: C, Caucasian; O, oriental.

Probe	Restriction enzyme	Fragment size	Frequency	Population	No. of chromosomes
pADH74	<i>Xba</i> I	4.4, 3.3	0.52, 0.48	C	112
			0.91, 0.09	O	22
pADH73	<i>Msp</i> I	12, 10	0.65, 0.35	C	46
			0.91, 0.09	O	24
pADH36	<i>Rsa</i> I	1.0, 0.5	0.78, 0.22	C	78
			0.18, 1.82	O	44

3 out of 20 tumours there was loss of expression of class I ADH, although class III ADH isoenzymes were present. In one sample, we were able to examine mRNA and to determine that although ADH mRNA was absent mRNA for α -fetoprotein was abundant. Analysis of DNA from tumours and adjacent normal liver tissue revealed that five of the samples were heterozygous at the ADH3 locus for the XBA I RFLP. In three of these samples there was evidence for loss of one of the alleles in tumour tissue. DNA from hepatomas and adjacent normal liver tissue was also examined for the EGF RFLP detectable with the restriction endonuclease *HincI*. Seven individuals proved to be heterozygous for the *HincI* EGF RFLP. In two cases, there was evidence of loss of one EGF allele in the tumour tissue, in a third case there was evidence of rearrangement of one of the EGF alleles. Two of the cases were heterozygous both at the ADH and at the EGF loci and showed evidence of allele loss or rearrangement at both loci (Smith *et al.*, 1985).

Buetow *et al.* (1987), recently described results of RFLP analysis in eight hepatomas. The chromosome 4q loci which they analysed were albumin (4q12-4q21) gIFN31.7, which is a gene encoding a γ -interferon-induced protein, (4q21), EGF (4q25-q27) and FGB, fibrinogen B (4q28-4q31). Four of the individuals were heterozygous for the EGF *HincI* RFLP, and in two of these cases there was evidence of allele loss in the tumour tissue. One of these tumours also showed evidence for loss of a gIFN 31.7 allele. These investigators were unable to detect loss of alleles for polymorphic markers located on human chromosomes 11, 14 and 18. One tumour did, however, show evidence of loss of alleles for a chromosome 13 polymorphic probe.

Taken together, results of our studies and those of Buetow *et al.* (1987) indicate that structural changes on human chromosome 4q in the region of the EGF and ADH genes may occur in a significant proportion of hepatomas. It will be of interest to determine if such structural changes are responsible for the changes in expression of the albumin- α -fetoprotein genes and for changes in the expression of ADH.

Further studies will need to be carried out to determine whether in these tumours the structural gene rearrangements can be related to integration of hepatitis B genome, since this virus has been shown to be the key factor in the aetiology of hepatoma (Sureau *et al.*, 1986). It is possible that changes in the expression of the EGF gene may constitute one of the primary defects which can lead to hepatoma formation since EGF is known to represent one of the most important growth factors for liver cells (Rubin *et al.*, 1982).

Evidence that sequence elements at the 3' ends play an important role in regulation of the tissue specific expression of ADH

Northern blot analysis of RNA from different tissues indicates that multiple forms of ADH mRNA occur in different tissue and at different times in development (Bilanchone *et al.*, 1986). The greatest size range of ADH mRNA species is found in adult liver where size classes of 2.6, 2.2, 1.9, and 1.6 kb occur. In adult liver all three class I ADH genes are expressed. In fetal liver, expressing primarily α -ADH, the 1.6 kb mRNA species predominates. In fetal intestine and lung, where γ - and β -ADH are expressed, respectively, but where ADH expression is very low, 2.6 kb size species of ADH mRNA predominate. Immunoprecipitation of adult liver mRNA translation products with polyclonal antibody followed by SDS/polyacrylamide gel electrophoresis revealed a single polypeptide 40000 Da in size. Results of nucleotide sequence analysis of β -, γ - and α -ADH cDNA clones, indicates that in all three transcripts a number of different potential polyadenylation sites occur

(Duester *et al.*, 1984; Ikuta *et al.*, 1986, Hedén *et al.*, 1986). In β -ADH at least five different polyadenylation sites occur.

Taken together these results indicate that in different tissues and at different stages of development, different polyadenylation sites are used in ADH transcripts. Of particular interest is the fact that in tissues where ADH expression is low, e.g. fetal intestine and lung, the largest size species of ADH mRNA predominates. There are a number of systems where mRNA transcript length has been shown to influence message stability and therefore gene expression. In the organism *Dictyostelium*, developmental regulation of gene expression has been shown to be effected through differences in mRNA stability (Mangiorotti *et al.*, 1985). Extended 3' untranslated regions in α -globin mRNA have been shown to be associated with decreased message stability (Higgs *et al.*, 1983).

Evidence that sequences at the 3' end of the mitochondrial ALDH gene influence gene expression

Electrophoretic studies of extracts of fetal tissue revealed that mitochondrial ALDH is absent or expressed only in very low amounts in fetal tissue (Hopkinson *et al.*, 1985). In screening 50 000 clones in a human fetal liver cDNA library with a mitochondrial ALDH cDNA clone, we isolated 27 clones with inserts of approximately 1.7 kb. Restriction mapping of these clones revealed that the clones matched mitochondrial ALDH cDNA clones from adult liver, except that they had extended 3' regions and contained an excess of 100-140 bases in this region (Baumann *et al.*, 1987). The frequency of mitochondrial ALDH clones in the fetal liver library was surprising in view of the absence of mitochondrial ALDH enzyme. Findings of restriction mapping of the mitochondrial ALDH clones in fetal liver indicate that gene expression may be altered because of differences at the 3' ends of the gene transcripts. We are currently carrying out sequence analysis to determine the exact differences in nucleotide sequences between fetal and adult mitochondrial ALDH clones.

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Molecular genetics of alcohol-metabolizing enzymes

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Remarkable racial differences of isoenzyme components of the ethanol metabolizing enzymes, i.e. the class I alcohol dehydrogenase [alcohol: NAD oxidoreductase (ADH), EC 1.1.1.1] and the aldehyde dehydrogenase [aldehyde: NAD oxidoreductase (ALDH), EC 1.2.1.3], in conjunction with the differences in alcohol sensitivity between Caucasians and Orientals, have been the subject of interest of biochemical and genetic studies in recent years. Liver ADH activity of about 90% of Orientals is severalfold higher than that of most Caucasians (Stamatoyannopoulos *et al.*, 1975), while approximately 50% of Orientals lack the activity of mitochondrial ALDH (ALDH₂) in their livers and other tissues (Harada *et al.*, 1978). The enzyme differences, mainly the absence of the active ALDH₂ component, and the consequent accumulation of acetaldehyde in the body, have been attributed to the high frequency of alcohol flushing in Orientals.

Alcohol dehydrogenase (class I ADH)

Human liver contains many ADH components (from 6 to 15 depending upon genotypes) which consist of homo- and hetero-dimers of three types of subunits, α , β and γ . The three subunits are controlled by three non-allelic genes, i.e.

Abbreviations used: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; RFLP, restriction fragment length polymorphism(s); CRM, cross-reactive material.

ADH₁ for α , ADH₂ for β and ADH₃ for γ (Smith *et al.*, 1971, 1972a,b). The ADH₂ and ADH₃ genes are polymorphic. The usual ADH₂¹ gene for the β_1 -subunit is common (about 90%) in Caucasians, while the frequency of the atypical ADH₂² gene for the β_2 -subunit is high (about 70%) in Orientals (Stamatoyannopoulos *et al.*, 1975). The two types of ADH₃ allele, i.e. ADH₃¹ and ADH₃², are common in Caucasians, while the frequency of ADH₃² is low in Orientals (Smith *et al.*, 1972a; Teng *et al.*, 1979).

The specific activity of atypical $\beta_2\beta_2$ enzyme is about 100 times higher than that of the usual $\beta_1\beta_1$ enzyme at the physiological pH (Yoshida *et al.*, 1981). The structural difference between the β_1 - and β_2 -subunit is a single amino acid substitution Arg → His at the 47th position of the subunit (Yoshida *et al.*, 1981; Ikuta *et al.*, 1985).

Complementary (c) DNAs and genes for the three subunits were cloned and the nucleotide sequences of the cDNAs were determined (Ikuta *et al.*, 1985, 1986). The complete amino acid sequences of the α -, β - and γ -subunits were deduced from their cDNA sequences. A high degree of resemblance (about 95%) was observed in their primary protein structures, coding cDNA sequences and even in their untranslated regions. The three genes for the α -, β - and γ -subunits were also similar to each other, i.e. the sizes were 10 ~ 15 kb and contained 9 exons. The three ADH genes most likely originated by gene duplication and subsequently diverged at the primate stage. Despite their general similarity, each subunit has a unique sequence in the vicinity of Cys-46 which ligates the catalytic Zn, i.e. α : Cys-Gly-Thr, β_1 : Cys-Arg-Thr, β_2 : Cys-His-Thr and γ : Cys-Arg-Ser (Ikuta *et al.*, 1986). This suggests that strong evolutionary pressures contributed to the divergency.

Based on their cDNA sequences, synthetic oligonucleotide probes which are specific for individual α -, β - and γ -mRNA were prepared (i.e. α probe: 3' TAGGGT GAGCGATAAGGA 5'; β probe: 3' CCCCAC-