Genetic analysis of two rat acetyltransferases

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Single copies of two closely related acetyltransferase genes were detected in Sprague-Dawley derived rat DNA by Southern blot analysis using gene-specific hybridization probes for the 3' end of the acetyltransferase coding regions. Sequence analysis of the two acetyltransferase genes showed that both had intronless, 870 bp coding regions and coded for 290 amino acid protein sequences that were ~85% homologous to one another. The calculated molecular weights were 33.4 and 33.9 kDa and the calculated isoelectric points 4.98 and 5.21 for AT1 and AT2, respectively. The inferred amino acid sequence of both the genes and cDNAs indicated that both rat acetyltransferases have cysteines at positions 44, 68 and 223 which have been conserved in all known vertebrate acetyltransferases. Transcripts for both AT1 and AT2 were detected in brain, colon, esophagus, heart, kidney, liver, lung, mammary gland, dorsal prostate, ventral prostate, salivary gland, seminal vesicles, small intestine, spleen, stomach, testes, urinary bladder and uterus of Sprague-Dawley rats by both Northern blot and RT-PCR analysis. A third gene with >80% sequence homology to codons 118-158 of acetyltransferase was also detected.

Introduction

The genotoxic effect of aromatic amines involves N-oxidation and conjugation to form O-esterified hydroxylamines that are capable of reacting covalently with nucleic acid (1). These reactive O-acetoxy metabolites can be formed either through the direct acetyl coenzyme A dependent O-acetylation of hydroxylamines (2) or through internal N,O-acetyltransfer of acetoxyhydroxamic acids (3). Strong evidence using the rat model supports the theory that metabolic activation of aromatic amines involves A^-oxidation followed by conjugation to form O-esterified hydroxylamines that may be converted to reactive metabolites and bind to DNA. These reactive metabolites can be formed either as the result of the direct acetylation of the reactive intermediates (i) by enzymes capable of producing the reactive metabolites or through internal MO-acetyltransfer and trans-acetylation (6). While multiple acetyltransferase proteins have been demonstrated for human (7,8), mouse (9) and hamster (10,11), only one acetyltransferase protein appears to be produced in rat liver (6). The objective of the present study was to establish through molecular biology techniques whether there is more than one acetyltransferase gene in the rat and, if so, whether the transcripts of more than one gene are produced. Knowledge of the sequence of acetyltransferase mRNA(s) can be expected to provide important information about the expression of acetyltransferase and the catalytic properties of proteins derived from this gene(s).

Materials and methods

Enzymes

Proteinase K, DNase-free RNase, SuperScript reverse transcriptase, E. coli RNase H, terminal deoxynucleotidyl transferase, murine leukemia virus (MuLV) reverse transcriptase, Hind III, Eco RI, Bam HI, Pst I, Eco RV and Pvu II were purchased from Gibco BRL Life Technologies (Gaithersburg, MD); AmpliTaq DNA polymerase from Perkin Elmer Cetus (Norwalk, CT); Gelase from Epicentre Technologies (Madison, WI); random hexadeoxynucleotide primers from Pharmacia LKB Biotechnology (Piscataway, NJ); Sequenase T7 DNA polymerase from United States Biochemicals (Cleveland, OH) and Exo^-Tfi DNA polymerase from Stratagene (LaJolla, CA).

Nucleotides

[alpha-32P]-dATP was purchased from New England Nuclear (Boston, MA) and [alpha-32P]-dCTP from ICN Biomedicals (Irvine, CA). Unlabeled deoxynucleotides were purchased from Perkin Elmer Cetus (Norwalk, CT).

Oligonucleotide primers

Synthetic oligodeoxynucleotides were synthesized by the Macromolecular Core Facility at Wayne State University with an Applied Biosystems Model 394 DNA/RNA synthesizer, using B-cyanoethyl phosphoramidite chemistry. Oligo version 3.4 software (National Biosciences, Hamel, MN) was used to optimize the primers for PCR amplification and DNA sequencing. Figure 1 shows the oligonucleotide primers used in this study. The sequences of the primers were derived as previously described for the hamster (10).

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Other materials

GlassMax DNA isolation spin cartridges were purchased from Gibco BRL Life Technologies (Gaithersburg, MD); NENSORPB Prep nucleic acid purification columns and Gene Screen Plus membranes from New England Nuclear (Boston, MA); Zeta Probe membranes from BioRad Laboratories (Richmond, CA); NuSieve low melting point agarose from BioRad Laboratories (Richmond, CA); molecular biology-grade glycogen from Boehringer Mannheim (Indianapolis, IN); and acetylated-bovine serum albumin was from New England Biolabs (Beverly, MA).

Animals

Male and female adult Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). They were maintained in polycarbonate cages with solid bottoms on layers of hardwood shavings in air-conditioned rooms at 22-24°C at 55% humidity on a 12 h light-dark cycle. Water and laboratory chow preparations (Harlan Teklad, Madison, WI) were ad libitum.

Isolation of DNA

DNA was purified by the method of Davis et al. (12). After phenol extraction, the DNA was precipitated with ethanol and dissolved in TE buffer overnight at 4°C.

Enzymatic amplification of DNA by PCR

PCR amplification was carried out as described (13,14), using 35 cycles of denaturation for 1 min at 94°C, annealing for 2 min at the average melting temperature for the two primers, and polymerization for 1 min/kb of template at 72°C. The melting temperatures for the oligo primers were calculated with
Northern blot hybridization analysis of RNA

Synthesis of hybridization probes

Probes were labeled with \( ^{32}P \)-dCTP by 20 cycles of PCR, using the given primers and 5 ng of the indicated template in 50 ul. The conditions were the same as for PCR, but with dATP, dGTP and dTTP each at 30 nM, and \( ^{32}P \)-dCTP at 3 nM and 600 mCi/nmol. Following synthesis, the labeled DNA was ethanol precipitated and heat denatured (14). The PCR primers used for synthesis of the rat acetyltransferase probes are as follows: 3' ATI: GSP2+ and AT1r876-; 3' AT2: GSP2+ and AT2/876-; central AT2: p3+ and p7-. The 3' AT1 and AT2 probes were 403 bp long; their sequences were mismatched by 11.2%. The central AT2 probe was 335 bp long and mismatched with ATI by 8.4%.

Isolation of RNA

The cesium chloride method of Chirgwin et al. (17) or the guanidine thiocyanate method of Chomczynski and Sacchi (18) was used to purify RNA. The RNA pellet was resuspended in 0.3 M sodium acetate, pH 6, ethanol precipitated, and redissolved in formamide to inhibit RNases (21). The hybridization probes were \( ^{32}P \)-labeled, and specific for either rat AT1 or AT2. The post-hybridization stringent wash was at 55°C.

PCR amplification of full-length acetyltransferase cDNAs for sequence analysis

The rapid amplification of cDNA ends (RACE) principle described by Ohara et al. (22) and Loh et al. (23) was used to PCR-amplify acetyltransferase cDNAs synthesized from polyadenylated liver RNA. For synthesis of the 3' end of acetyltransferase cDNAs, RT was primed with oligo dT; the 5' end was primed with GSP2-, Gibco BRL Life Technologies 5' and 3' RACE kits and protocols were used.

Analysis of acetyltransferase transcripts by RT-PCR

Total cDNA was synthesized from polyadenylated RNA from the indicated tissues using MuLV reverse Transcriptase essentially as in the previous section, but primed with 3 μM random hexanucleotides. Using primers which were both specific for either rat AT1 (AT1/91+ and AT1r/876-) or AT2 (AT2/91+ and AT2/876-), the indicated acetyltransferase cDNAs were then selectively amplified from the total cDNA. The PCR thermal cycle was (95°C for 1 min, 45°C for 2 min, and 72°C for 1 min) for the first cycle, then (95°C for 1 min, 53°C for 2 min, and 72°C for 1 min) for the subsequent 24 cycles, followed by 72°C for 5 min. The amplified cDNAs were analyzed by agarose gel electrophoresis to evaluate the size and yield. The specificity of the PCR products was determined by (i) sequence analysis, or (ii) PCR re-amplification with either the same or non-homologous primers (e.g. AT1 re-amplified with AT1 or AT1 reamplified with AT2 primers).

DNA sequencing

Single-stranded DNA was sequenced by Sanger's dideoxy method (24) using \( ^{32}P \)-dATP as outlined by United States Biochemical (25). Double-stranded DNA was sequenced by cycle sequencing (26,27) as outlined by Stratagene (Cyclist Exo + Pfu DNA Sequencing Kit). The cycle sequencing reactions were analyzed by polyacrylamide gel electrophoresis and autoradiography for single-stranded DNA sequencing.

Computer analysis of protein and nucleic acid sequences

The University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package (Version 7.2, release of 1992) was used, which was updated 4/1993 with the following GCG Support databases (release in
Fig. 3. Sequence divergence of rat acetyltransferase cDNAs. Nucleotides -16 through +25 are shown for rat AT2 cDNA (GenBank no. U17261), where +1 is the first base of the start codon. For the three other rat acetyltransferase cDNAs, individual nucleotides are shown where sequences differ; hyphens indicate the sequences are identical. ATb-7 and ATa-1 are from Ebisawa et al. (32); rat AT1 (GenBank no. U17260).

Results

Sequence analysis of acetyltransferase genes and cDNAs

Evolutionarily conserved sequences from chicken (28), rabbit (29), and human (30) liver acetyltransferases were used to design two primers: p3+ (codons 118-126) and p7− (codons 222-230). The 3′+7′− PCR amplified DNA segment gave a single sequence, which was 93% homologous to the mouse AT2 (31) and 90% homologous to hamster AT2 (10). To obtain the entire coding sequence of rat acetyltransferase genes, rat cDNAs were RT-PCR amplified from liver polyadenylated RNA, using the 5′ and 3′ RACE techniques. The major 5′ products were 750 and 1000 bp long. The sequence obtained for codons 1-150 of the 1000 bp cDNA was more homologous with the corresponding region of AT1 than AT2 from either mouse (93.8% versus 83.3%) or hamster (88.4% versus 83.3%), and was, therefore, tentatively presumed to be the first half of the rat AT1 open reading frame. In contrast the 750 bp product contained an acetyltransferase cDNA which was >91% homologous with the first 150 codons of mouse and hamster AT2, while it differed from the AT1s of these rodents by ~17%. Therefore, this appeared to be a rat AT2 cDNA. The major 3′ RACE acetyltransferase cDNAs were 700, 1200, and 1600 bp long. The coding sequences of all of these were identical, and appeared to be AT1 cDNAs by comparison of sequence homologies with other species.

To confirm these data and to complete the coding region of the 3′ end of the second rat acetyltransferase gene, the cDNAs for the two acetyltransferases were individually PCR amplified from a rat liver total cDNA library prepared using random-hexanucleotide priming. The gene-specific primer combination used for PCR amplification of AT1 was AT1r/876− and AT1/91+; for AT2 it was AT2/876− and AT2/91+. The results of this analysis provided the sequence for codons 151–290 for rat AT2 cDNA, and confirmed the AT1 cDNA sequence obtained previously. AT1 and AT2 genes were then amplified from rat genomic DNA with primers S5′ + and AT1/876− or AT2/876− and sequenced. The coding regions obtained matched those of the two acetyltransferase transcripts. Both AT1 and AT2 had intronless, 870 bp coding regions. The coding sequences are identical to those that have been published by Ebisawa et al. for two acetyltransferase cDNAs obtained from the Wistar rat pineal gland (32) during the preparation of this report. Where comparisons could be made between the non-coding flanking sequences in these two studies, the only differences in the two AT2 sequences were in 7 of 8 bases that were immediately 5′ to the putative splice site 6 bases upstream of the start codon (Figure 3). There were no differences found in the sequences flanking the two AT1 genes.

Fig. 4. Northern blot analysis of acetyltransferase transcripts in rat tissues. Northern blot hybridization was carried out on 20 μg aliquots of polyadenylated mRNAs. [32P]-labeled probes for AT1 and for AT2 were used. The exposure times for the autoradiograms were 2 to 3 weeks. The AT2 portion is a composite of two different exposures because of the great diversity of transcript quantity between the small intestine and colon from the other tissues shown. Approximate sizes (kb) are given on the left.

Fig. 5. PCR amplification of rat liver specific AT1 or AT2 cDNAs. The entire coding region of AT1 or AT2 cDNAs was PCR amplified from total rat liver cDNA (made from polyadenylated liver RNA with randomly-primed reverse transcriptase). The primers used for AT1 were 5′FS+ and AT1/876−, and those used for AT2 were 5′FS+ and AT2/876−. The PCR products were approximately the expected 888 bp, and confirmed to be the expected acetyltransferase cDNAs by sequence analysis (data not shown). To further test the gene specificity of the PCR primers used, these amplified cDNAs were diluted 100-fold and reamplified as before with the indicated primers, then analyzed by electrophoresis through 1.5% agarose/ethidium bromide gels. The primers used were either nested primers with the same specificity (lanes 2 and 5) or the primers for the opposite acetyltransferase gene, which gave no reamplification of the input template (lanes 3 and 4). Lane 1 contained DNA size standards (100 bp ladder), where the 600 bp marker is shown on the left. These results indicate that transcripts for both AT1 and AT2 were present and that amplification of the target rat acetyltransferase cDNA is highly specific under these conditions.

AT2    CAGCAG CTCTGAAACC ATGGACATGT AGCATACTT TGAAAA
ATb-7  --ATT - ACTG-------- C- C- C- C- C- C- C-
AT1    --ATT - ACTG-G-G-- C- C- C- C- C- C- C-
Ata-1  --ATT - ACTG-G-G-- C- C- C- C- C- C- C-

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DNA restriction analysis of acetyltransferase gene copy number

To estimate the number of acetyltransferase genes in the haploid genomes of the rat, Southern blot analyses were carried out on cellular DNAs that had been cut with six different hexanucleotide restriction endonucleases. Statistically, these were expected to give DNA fragments averaging ~4 kb, and to give one hybridization signal per gene in most cases. This is because such fragments should be large enough to leave intact the regions to which the acetyltransferase probes hybridize, but small enough to contain only one of multiple acetyltransferase genes even when they are closely linked (e.g. mouse AT1 and AT2 are separated by only ~9 kb) (31).

As shown in Figure 2, hybridization with probes specific for AT1 (panel A) showed a single AT1 gene while AT2 probes (panel B) indicated that two major acetyltransferase bands were present. Cross-hybridization of the AT2 probe to the AT1 acetyltransferase gene appears to account for the second band in each lane. Interestingly, the ‘central’ acetyltransferase probe (panel C) gave an additional band which was not coincident with either of the acetyltransferase genes. By inference, this has >80% sequence similarity with codons 118–158 of AT2, and remains unidentified at present.

Northern blot analysis of tissue-specific acetyltransferase mRNA levels

Polyadenylated mRNAs extracted from rat brain, liver, kidney, spleen, lung, heart, salivary gland, esophagus, stomach, small intestine, colon, mammary gland, uterus, urinary bladder, ventral prostate, dorsal prostate, seminal vesicles, and testes were analyzed for acetyltransferase transcripts by hybridization of Northern blots to probes specific for either AT1 or AT2. Sex-nonspecific tissues were either from male or female rats for larger tissues such as the liver, but for less abundant tissues such as the urinary bladder the tissues from male and female rats were pooled. The apparently low copy number of acetyltransferase transcripts required prolonged exposure times for autoradiography, which resulted in high backgrounds. The data obtained with representative tissues are shown in Figure 4. Each tissue showed an ~1.7 kb transcript using the AT2 probe and ~1.7 kb and ~2.1 kb transcripts with the AT1 probe. Since the 1.7 kb AT1 and AT2 bands are coincident, cross-hybridization of the AT1 probe to AT2 transcript cannot be ruled out. The highest abundance of transcripts was found in brain, small intestine and colon and the lowest abundance in heart.

Selective RT-PCR amplification of tissue-specific AT1 or AT2 transcripts

Randomly-primed cDNA libraries were prepared from rat liver polyadenylated RNA and then amplified with a generic primer 5′fs+, that efficiently amplified both acetyltransferase cDNAs under the conditions used, plus a primer which was gene-specific for either AT1 (AT1r/876−) or AT2 (AT2/876−). The PCR products were of the size (~888 bp) expected for this region of the acetyltransferase cDNAs. To confirm the acetyltransferase cDNA-specificity of PCR amplification, gene-specific PCR amplification of these acetyltransferase cDNAs was then carried out using nested primers which were both specific for either AT1 (AT1f/91+ and AT1r/876−) or AT2 (AT2f/91+ and AT2r/876−) (Figure 5). That these PCR products were exclusively AT1 or AT2 was then demonstrated by the inability of nested primers to reamplify the initial PCR products unless the homologous primers were used (i.e. AT1 primers for AT1 and AT2 primers for AT2). The PCR products were of the expected size (~786 bp).

Analysis of tissue-specific acetyltransferase mRNA by RT-PCR

To verify the tissue distribution information obtained with the Northern blot analysis, randomly primed cDNA libraries were prepared from polyadenylated RNAs isolated from each of the 18 tissues used in the Northern blot analysis, PCR-amplified with 5′fs+ and either AT1r/876− or AT2/876−. To show specificity, the PCR products were then reamplified using nested AT1 and AT2 specific primers (AT1: AT1f/91+ and AT1r/876− and AT2: AT2f/91+ and AT2r/876−). The products were analyzed by electrophoresis through a 1.5% agarose/ethidium bromide gel. Lane 1 contained DNA size standards (100 bp ladder), where the 600 bp marker is shown on the left. The results indicate that transcripts for both AT1 and AT2 were present in all of the tissues analyzed.

Discussion

Two rat acetyltransferase genes have been identified and sequenced from Sprague–Dawley derived rats. These genes have been designated AT1 and AT2 based on homologies with hamster AT1 and AT2 gene sequences of 87.7% and 90%, respectively. The rat sequences show 84.7% homology to one another. The coding sequences are identical to those that have been published by Ebisawa et al. for two acetyltransferase cDNAs obtained from the Wistar rat pineal gland (32) during
the preparation of this report. Where comparisons could be made between the non-coding flanking sequences in these two studies, the only differences in the two AT2 sequences were in 7 of 8 bases that were immediately 5' to the putative splice site 6 bases upstream of the start codon (Figure 3). No differences in the sequences flanking the two AT1 genes were detected.

In agreement with the report from Ebisawa et al. (32), Southern blot analysis using gene-specific probes for the 3' end of the acetyltransferase coding region detected a single AT1 and AT2 gene. In Figure 2, the second band in panel B is thought to be due to cross hybridization of the AT2 probe with the AT1 gene. However, a central probe spanning codons 118–158 hybridized with an unknown third sequence which appeared to have >80% sequence homology with this region. Similar observations have been made with the hamster genome (10).

Both rat AT1 and AT2 have intronless coding regions of 870 bp that code for 290 amino acids, as do the reported sequences for acetyltransferases from human (30), hamster (10,33,34), mouse (31), rabbit (35,36) and chicken (28). The only known exception is a chicken liver acetyltransferase which has 287 amino acids (37). Both rat acetyltransferase sequences have cysteines at positions 44, 68 and 223, as do all known vertebrate acetyltransferase sequences. The calculated molecular weights were 33.4 and 33.9 kDa; the calculated isoelectric points were 4.98 and 5.21 for AT1 and AT2, respectively.

Northern blot analysis and RT-PCR techniques demonstrated the presence of mRNA transcripts for both rat AT1 and AT2 in brain, liver, kidney, spleen, lung, heart, salivary gland, esophagus, stomach, small intestine, colon, mammary gland, uterus, urinary bladder, ventral prostate, dorsal prostate, seminal vesicles, and testes. Ebisawa et al. have demonstrated the presence of AT1 and AT2 mRNAs in rat liver, kidney, brain, pineal gland and heart by use of Northern blots that disclosed the presence of multiple species of AT1 mRNA consistent with the present report and a single weak signal for AT2 (32). Such widespread distribution is consistent with studies that have shown acetyltransferase activities in rat tissues (3), activities and transcripts in eight hamster tissues studied (10) and activities in all but two of 35 mouse tissues assayed (38). In the mouse study, seminal vesicles showed no acetyltransferase activity while in the present study rat seminal vesicles showed transcripts for both AT1 and AT2. Similar, but less extensive, data are available for a wide range of tissues from many species (39).

Two possibilities exist for the presence of a mRNA transcript for rat AT2 and the absence of evidence for an AT2 protein: (i) the transcript may be so unstable that translation is precluded, or (ii) the transcript is translated but the protein is either inactive, unstable, or present at too low a steady state level for detection. Sequence analysis of the 3' untranslated region of wild type human NAT1 (40) shows similarity to eukaryotic mRNAs with high turnover rates, i.e. multiple copies of AAT, ATA, and TAA motifs. These regions are thought to disrupt the association of poly(A) binding proteins (41-43). Vatsis and Weber (40) also found a mutation in the polyadenylation signal (AAATTTA) in one mutant NAT1 gene found in Caucasians. Non-polyadenylated mRNAs are more rapidly degraded (43). Sequencing of the non-coding regions of the rat AT2 sequence may provide insight into the potential for sequence-influenced transcript stability and the translation efficiency of the transcript. In situ hybridization using rat tissues have indicated that AT1 transcripts are present in a wide range of tissues and that they exhibit highly specific distributions within distinctive cell types (44). AT2 transcripts are found in all tissues where AT1 transcripts are detected, but their levels are much less abundant (44). Cloning of the sequences of rat AT1 and AT2 into expression vectors has produced catalytically active rat acetyltransferases in both mammalian (32) and bacterial cells (45). These studies should permit catalytic and turnover studies that will aid in clarifying whether the apparently low level of AT2 activity results from inherent protein instabilities. Furthermore, the ability to express these enzymes may help clarify their role and regulation in the metabolism of endogenous substrates that is implicit in their evolutionary conservation in vertebrates. Such experiments are in progress.

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