Mapping of Genes for Cytochromes P-450b, P-450e, P-450g, and P-450h in the Rat

DEMONSTRATION OF TWO GENE CLUSTERS WITH P450-b,e LOCALIZED ON CHROMOSOME 1*

(Received for publication, October 7, 1986)

Arfaan Ramperesa and Frederick G. Walz, Jr.‡

From the Department of Chemistry, Kent State University, Kent, Ohio 44242

Inbred ACI, WF, and RCS rats having characteristic markers for albinot (e), hemoglobin β-chain (Hbb), and pink-eyed dilution (p) on chromosome 1 and expressing variants for hepatic cytochromes P-450b, P-450e, P-450g, and P-450h were used in genetic mapping studies for these hemoproteins. The results of WF × (ACI × WF)F1 and RCS × (WF × RCS)F1 backcrosses revealed the existence of two gene clusters designated the P450-b,e and P450-g,h loci. The linkage map P450-b,e-p-c-Hbb on rat chromosome 1 was demonstrated and found to be congruent with Coh(P450-b,e)-p-c-Hbb on mouse chromosome 7. P450-g,h is not linked with P450-b,e and the other markers tested on rat chromosome 1. It appears that close genetic linkage, rather than common functional/regulatory properties, typify members of cytochrome P-450 families/subfamilies.

Mammalian cytochromes P-450 constitute a superfamily of membrane-bound enzymes that function as terminal monooxygenases in the metabolism of a broad variety of endogenous and exogenous compounds (1, 2). These b-type hemoproteins have been extensively studied in the laboratory rat (Rattus norvegicus) and more than a dozen isozymic (i.e. nonallelic) forms have been purified from hepatic microsomes (3). Every isozyme purified to date contains a single polypeptide (50 ± 10 Kd) and has been shown to have a characteristic but broad substrate specificity in vitro.

Rat-hepatic cytochromes P-450b and P-450e are co-induced by phenobarbital (as well as other agents (4)) and share 97% homology in their amino acid sequences (5, 6). Electrophoretic/genetic studies demonstrated the nonallelic relationship of these extremely homologous enzymes and revealed the existence of electromorphic variants for both forms that were expressed in a codominant fashion (7). In addition, genes for chromosomes P-450b and P-450e (i.e. P450-b(e) and P450-e(e), respectively) were shown to be tightly linked on an autosomal chromosome (7).

Studies employing recombinant DNA methods determined that at least four more genes/pseudogenes characterize the cytochrome P-450b,e family in the rat (10, 11). Furthermore, the results of experiments using a cDNA containing rat cytochrome P-450b information indicated that corresponding genes for the cytochrome P-450b,e family in the mouse are all linked with the Coh (coumarin hydroxylase) locus on chromosome 7 (12). Since rat chromosome 1 (formerly linkage group I (13)) and mouse chromosome 7 have similar linkage maps (e.g. for p (pink-eyed dilution), c (coat color), and Hbb (hemoglobin β-chain) loci) (14), it would be of interest to test whether the P450-b,e locus occupies a position on rat chromosome 1 that is analogous to that for Coh on mouse chromosome 7.

Hepatic cytochromes P-450g and P-450h in the rat are members of a different, immunochemically related family that also includes cytochromes P-450f, P-450i, and P-450k (15). These five isozymes are also characterized by homologous N-terminal sequences (16); however, recent evidence suggests that cytochromes P-450f and P-450k are members of a more limited subfamily (17). Cytochromes P-450g and P-450h are both male-specific and their levels increase dramatically during the course of pubescence (18, 19). Three electrophoretic variants of cytochrome P-450h were discovered (20) and an apparently cis-acting regulatory polymorphism was found to characterize cytochrome P-450g (21). It was also shown that corresponding genes for these polymorphisms (i.e. P450-h(e) (20)) and P450-g(r) (21)) are autosomal and express alleles in a codominant fashion. Considering that P450-b(e) and P450-e(e) are clustered in the rat as are orthologous genes in the mouse (see above) it would be interesting to determine whether another cytochrome P-450 gene family (e.g. that containing information for cytochromes P-450g and P-450h) is also closely linked.

In order to map the P450-b,e locus and to determine the linkage relationships for P450-g(r) and P450-h(e), we surveyed 28 inbred strains of rat for appropriate markers (22). In this article we describe the results of genetic studies using inbred ACI, WF, and RCS rats that express variants for P450-b(e), P450-e(e), P450-g(r) and P450-h(e) in addition to markers for p, c, and Hbb on chromosome 1. We demonstrate that the P450-b,e locus maps to chromosome 1 at a position that is analogous to that for Coh on mouse chromosome 7. In addition, P450-g(r) and P450-h(e) are shown to be tightly linked and to segregate independently from all the markers tested on chromosome 1.

MATERIALS AND METHODS

Genotypes, Breeding, and Treatment of Rats—Inbred ACI/NHsd and WF/Hsd strains were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), whereas inbred RCS/N rats were provided by the Small Animal Section of the National Institutes of Health (Bethesda, MD). ACI rats are brown, agouti, and Irish hooded (C/CA/
A, Hb' (23), express the b-type hemoglobin chain variant (Hbb' \(\rightarrow\) Hbb) (24) and are characterized by the hepatic cytochrome P-450 genotype; P450-b(e) \(\rightarrow\) P450-\(b(e)^\prime\), P450-e(e) \(\rightarrow\) P450-\(e(e)^\prime\), P450-g(r) \(\rightarrow\) P450-\(g(r)^\prime\), P450-h(e) \(\rightarrow\) P450-\(h(e)^\prime\) (22). WF rats are albino (c/c), express the a-type hemoglobin chain variant (Hbb'/Hbb) (24) and possess the hepatic cytochrome P-450 genotype; P450-b(e) \(\rightarrow\) P450-\(b(e)^\prime\), P450-e(e) \(\rightarrow\) P450-\(e(e)^\prime\), P450-g(r) \(\rightarrow\) P450-\(g(r)^\prime\), P450-h(e) \(\rightarrow\) P450-\(h(e)^\prime\) (22). RCS rats are tan-hooded and pink-eyed (C/C,a/a,h/h,p/p) (25), express the b-type hemoglobin chain variant (Hbb'/Hbb) and carry the cytochrome P-450 genotype, P450-b(e) \(\rightarrow\) P450-\(b(e)^\prime\), P450-e(e) \(\rightarrow\) P450-\(e(e)^\prime\), P450-g(r) \(\rightarrow\) P450-\(g(r)^\prime\), P450-h(e) \(\rightarrow\) P450-\(h(e)^\prime\) (22).

Males and females of a given strain were used in five crosses between ACI and WF rats and three crosses of WF and RCS rats. Progenies of these crosses are designated (ACI \(\times\) WF)F1 and (WF \(\times\) RCS)F1, respectively, and these were used in the backcrosses, WF \(\times\) (ACI \(\times\) WF)F1 and RCS \(\times\) (WF \(\times\) RCS)F1. \(x^2\) values were calculated for allelic segregation (\(x_1\)) and linkage (\(x_2\)) and corresponding probabilities (\(p_1\) and \(p_2\), respectively) were interpolated.

Maintenance of all rats and phenobarbital treatment of males were as described (20), whereas females were treated with 0.1% phenobarbital in their drinking water for 5 days. Sexually mature rats (100–130 days) were starved 18 h before killing by decapitation. Prior to drug treatment, blood samples were taken from the tails of lightly etherized rats and collected in sterile tubes containing K3EDTA. After killing, total hepatic microsomes were prepared in the absence of protease inhibitors to facilitate the analysis of cytochrome P-450 variants (20).

Analytical Procedures—Two-dimensional IF/NaDodSO4 electrophoresis was used to analyze phenotypes for cytochromes P-450, P-450e, P-450g, and P-450h (7, 20, 21). b-Chain variants of hemoglobin were resolved from red blood cell lysates in 12% polyacrylamide slab gels (160 × 180 mm) containing 8 M urea, 5% acetic acid (v/v), and 0.9% Triton X-100 (w/v) (26). Briefly, isotonically washed cells were packed by centrifugation (1000 × g, 10 min) and lysed in 0.05 M Tris-Cl, pH 8.3. Membranes were removed by centrifugation (20,000 × g, 30 min), and the supernatant solution (lysate) was stored at −70 °C. One volume of lysate was diluted with 2 volumes of sample buffer (27) and 50-μl samples were electrophoresed. The resolution of a and b variants of the hemoglobin b-chain is shown in Fig. 1 where red blood cell lysates from WF, (ACI \(\times\) WF)F1 and ACI rats were electrophoresed in the left, center, and right lanes, respectively. The clear distinction of one-band (homozygous) and two-band (heterozygous) phenotypes in adjacent bands of a slab gel facilitated the analysis of these variants among the backcross progenies used in the present study.

RESULTS AND DISCUSSION

Five crosses of ACI and WF rats produced 36 progeny. As expected both male and female (ACI \(\times\) WF)F1 animals were found to be heterozygous at the Hbb locus (Hbb'/Hbb, see Fig. 1, center lane) and were pigmented (C/c) like their ACI parents. Hepatic microsomes from individual (ACI \(\times\) WF)F1 rats were analyzed by IF/NaDodSO4 electrophoresis which indicated that they were also heterozygous for P450-b(e), P450-e(e), P450-g(r), and P450-h(e) (the latter two genes are only expressed in males). Results for (ACI \(\times\) WF)F1 males are exemplified in Fig. 2B which indicates the presence of two electrophoretic variants for P450-b(e), P450-e(e), and P450-g(r), whereas the level of cytochrome P-450-g(e) is clearly distinct from that in WF rats (exemplified in Fig. 2A). These F1 heterozygotes were reciprocally backcrossed to the WF strain (12 mating pairs) which produced 122 males (60 males, 60 females). Progenies were screened for pigmentation (65 albino, 57 colored; \(p_a = 0.48\) and hemoglobin b-chain variants (66 Hbb'/Hbb, 56 Hbb'/Hbb; \(p_a = 0.38\). Hepatic microsomes from each member of this backcross generation were prepared and subjected to IF/NaDodSO4 electrophoretic analysis of cytochrome P-450 variants. Only four male-phenotypes for cytochromes P-450b, P-450e, P-450g, and P-450h were ob-

\[2\] The abbreviations used are: NaDodSO4, sodium dodecyl sulfate; IF, isoelectric focusing; K3EDTA, tripotassium ethylenediaminetetraacetate.

FIG. 1. Acid-urea-Triton X-100 gel electrophoretograms of red blood cell lysates from WF, ACI, and ACI \(\times\) WF)F1 rats. Electrophoresis was conducted from top to bottom. Allelic hemoglobin b-chain variants are resolved in the middle portion of the gel and designated Hbb' and Hbb. Left lane, WF; center lane, (ACI \(\times\) WF)F1; right lane, ACI. The hemoglobin variant (Hbb') shown in right lane also characterizes RCS rats. Gels were stained with Coomassie Blue. Other conditions are under "Materials and Methods."
in equal proportions (pL = 0.38). These observations are consistent with our previous report that genes for cytochromes P-450b and P-450e are tightly linked (7). Likewise, no recombinants were observed for P450-b(e) and P450-h(e), whereas both parental classes appeared in equal proportions (pL = 0.45). From this analysis we conclude that P450-b(e) and P450-h(e) are also expressed in males. However, when P450-b,e and P450-g,h loci were tested for independent segregation, both parental and recombinant types were found in essentially equal proportions (pL = 0.62, see Table I) indicating that these two clusters of cytochrome P-450 genes are not linked.

In addition, the segregation of P450-g,h with c (28 recombinants, 32 parental) and Hbb (28 recombinants, 32 parental) indicated that this cluster of cytochrome P-450 genes is also not linked to markers on chromosome 1. On the other hand, the segregation of P450-b,e with c (41 recombinants, 81 parents, pL < 0.01) and with Hbb (48 recombinants, 74 parents; pL < 0.05) and for the presumed gene order, P450-b,e-c-Hbb, no double recombinants were observed. Since the alternative order c-Hbb-P450-b,e would have yielded seven double recombinants and no single recombinants of a given class (see Table II), it is most likely that the proposed order is correct.

The location of P450-b,e on rat chromosome 1 was further substantiated in studies on its linkage with the p locus. RCS rats carry a recessive mutation (p/p) at this locus (25) and are homozygous for P450-b(e)7 and P450-e(e)9 (22), whereas WF rats are heterozygous for P450-b(e)5 and P450-e(e)2 (25). RCS rats are characterized by a dominant allele at the p locus (P/P). In addition, WF and RCS rats express a and b variants, respectively, for the hemoglobin β-chain. Therefore, segregation analysis of backcross progeny of RCS × (WF × RCS)F1 rats can be used for linkage analysis of p with P450-b,e and Hbb.

RCS and (WF × RCS)F1 rats were reciprocally backcrossed with albino WF rats (c/c) yielding 12 F1 progeny all of which were black-eyed, black (non-agouti)-hooded animals. This result indicates that WF rats have dominant alleles at the p locus and, therefore, the pigmentation/coat genotype (c/c,a/a,h/h,P/P) (20)). The observation that ACI and all (ACI × WF)F1 rats had the same pigmentation and coat supports the assignment of recessive alleles at the a and h loci for WF rats since ACI rats are dominant (A/A,h/h) at these loci (23).

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**TABLE I**

<table>
<thead>
<tr>
<th>Segregating alleles for P450-b(e), P450-e(e), P450-g(r), and P450-h(e) among progenies of WF × (ACI × WF)F1 backcrosses</th>
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<tr>
<td>Segregating genes Class Genotype No.</td>
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<tr>
<td>---------------------------------------------------------------</td>
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<tr>
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<td>P450-g(r)/P450-h(e)</td>
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<tr>
<td>Recombinant P450-g(r) P450-h(e) 16</td>
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*P450-g(r) and P450-h(e) are only expressed in males.

* Segregation of these genes is the same as that for P450-e(e) and P450-h(e).
TABLE II

Segregation of alleles for P450-b,e, p, c and Hbb in backcross progenies using WF, ACI, and RCS rats

Frequency of recombination ± S.E.: c-Hbb, 7/122 = 0.057 ± 0.021; P450-b,e-c, 41/122 = 0.336 ± 0.043; P450-b,e-Hbb, 77/189 = 0.407 ± 0.036 (p< 0.05); P450-b,e-p, 7/67 = 0.104 ± 0.037; p-Hbb, 24/67 = 0.358 ± 0.058 (p< 0.05).

<table>
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<th>Class</th>
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<th>RCS × (WF × RCS)/F1</th>
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<td>Genotype</td>
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<td>Single recombinant&lt;sup&gt;+&lt;/sup&gt;</td>
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*Abbreviations for alleles: b<sup>e</sup>,<sup>e</sup> = tightly linked haplotype for P450-b<sup>e</sup>-b<sup>e</sup>-e<sup>3</sup> and P450-e<sup>3</sup>-e<sup>3</sup>; b<sup>e</sup>,<sup>e</sup> = tightly linked haplotype for P450-b<sup>e</sup>-b<sup>e</sup>-e<sup>3</sup> and P450-e<sup>3</sup>-e<sup>3</sup>.

<sup>+</sup> For a given alternative gene order (i.e. c-Hbb-P450-b<sup>e</sup>-b<sup>e</sup>-c considering WF × (ACI × WF)/F1 progeny or p-P450-b<sup>e</sup>-b<sup>e</sup>-Hbb considering the RCS × (WF × RCS)/F1 progeny), this single recombinant class would be exchanged with the double recombinant class.

We calculated the apparent distances (cM) between P450-b<sup>e</sup>-b<sup>e</sup>, p, c and Hbb on rat chromosome 1 using the frequencies of recombination in Table II and an appropriate mapping function (28). These distances are shown in Fig. 3 where they are compared with literature values for some of these loci as well as those for warfarin resistance (i.e. War for the mouse and RW for the rat), lactate dehydrogenase (i.e. Ldh-1), glucosephosphate isomerase (i.e. Gpi) and peptidease-4 (i.e. Pep-4).

P450-b<sup>e</sup> in the mouse is linked with Coh (<4.4 cM at a 95% confidence level) (12). Therefore, a virtually complete congruency exists for the positions of P450-b<sup>e</sup>, p, c and Hbb on rat chromosome 1 and mouse chromosome 7. However, the interval separating p from Coh on mouse chromosome 7 contains the linkage group Gpi-Pep-4-Ldh-1 (see Fig. 3), whereas in the rat this group is located on chromosome 7 (31). Interestingly, a cDNA coding for presumably orthologous cytochrome P-450b/e in the human was used to assign a cytochrome P-450 gene family to human chromosome 19 in a region close to Gpi and Pep-4 (i.e. Pep-D) (32), whereas the human gene corresponding to c (i.e. Tyr = tyrosinase) is linked with Hbb and Ldh-1 (i.e. LDH-A) in the order LDH-A-Tyr-Hbb on chromosome 11 (33). Therefore, these findings indicate that a given linkage conservation of P450-b<sup>e</sup> may or may not overlap between different species. Nevertheless, it is noteworthy that genes composing the P450-b<sup>e</sup>-e<sup>3</sup> family remain clustered in the rat, mouse, and human.

Implications and Biological Significance—At this time it appears that all known closely related cytochrome P-450 gene families exist as clusters and in several cases the linkage distances between individual members have been determined. A tandem arrangement of two homologous pseudo and genuine genes for human adrenal steroid 21-hydroxylase on chromosome 6 has been demonstrated (34, 35) and corresponding linked genes have been detected on mouse chromosome 17 (36). Likewise, portions of two homologous genes for testosterone 15a-hydroxylase on mouse chromosome 7 were found in a 30-kilobase-pair fragment of cloned genomic DNA (37). Furthermore, mouse cytochromes P-450 and P-450, which are orthologous with rat cytochromes P-450c and P-450d, respectively, have amino acid sequences that are strongly homologous and their genes are separated by <16 cM on chromosome 9 (38). In addition, the results of the present study indicate in combination with those previously reported (7) yielded no recombinants between rat P450-b<sup>e</sup>-e<sup>3</sup>-e<sup>3</sup> in a total of 225 tests which gives an upper limit of 1 cM separating these loci on chromosome 1 at a 90% confidence level. Finally, the absence of any recombinants between P450-g(r) and P450-h(e) among 60 male backcross progeny in the present work indicates that there is a 90% probability that these genes are separated by <3.7 cM. Therefore, results to date for the genes composing the cytochromes P-450c,d, P-450b,e, and P-450g,h families are consistent with their being tandemly linked as was found for the adrenal steroid 21-hydroxylase and testosterone 15a-hydroxylase families. The future applications of novel methods to establish molecular distances between genes (i.e. measured in base-pairs approximating the equivalent of 1 cM (39)) could elucidate the precise position of cytochrome P-450 genes in a given cluster.
Members of a given cytochrome P-450 family/subfamily in the rat have been characterized by overlapping but distinct catalytic profiles (40–43). Likewise, the overall ontogenetic and/or inductive controls for specific genes in the cytochromes P-450b,e (44, 45), P-450c,d (4, 46), P-450f-i,k (19, 39), and P-450PCN (47) families are apparently unique. Therefore, at this time, a clustered gene organization rather than a common regulation or function appears to typify cytochrome P-450 families. The existence of cytochrome P-450 gene families in clustered arrays might explain, in part, the prevalence of gene conversions that have been evidenced among members of either the cytochromes P-450b,e (48, 49), P-450c,d (47), or P-450PCN (47) families (50).

Orthologues for cytochromes P-450 in the mouse have not been definitively characterized. Nevertheless, the mouse hepatic isozyme “C”-P45016α (51) shares the following properties with rat cytochrome P-450b: (a) high specific activity for testosterone 16α-hydroxylation (51), (b) constitutive, male-specific expression in adult animals being subject to regulation by growth hormone (19, 52), and (c) partial but discriminative N-terminal sequence homology (53). It is interesting that a cDNA for “C”-P45016α also exhibited coding sequence homology with rat cytochrome P-450b (53) which suggests that the gene for this hemoprotein might be linked with Coh (cytochrome P450-b,e) on mouse chromosome 7 (see above). The present study indicates that P450-b,e and P450-g,h are not linked in the rat and, in view of the linkage conservations discussed above, it is possible that P450-g,h is linked with Gpi and/or Pep-4 on rat chromosome 7. If indeed P450-b,e and P450-g,h are linked in the mouse but not in the rat then the evolution of these families (e.g., by gene conversions) might have followed considerably different courses after these rodent species diverged.

Acknowledgment—The assistance of Alex Nowicki in some experiments is gratefully acknowledged.

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