Isolation and Characterization of Genomic Clones for Two Chicken Phenobarbital-inducible Cytochrome P-450 Genes*

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A cDNA clone specific for a chicken phenobarbital-inducible cytochrome P-450 was used to screen a chicken genomic library. Twenty-nine clones were isolated, restriction mapped, and divided into two non-overlapping groups. The cDNA clone hybridized to 12 kilobases of DNA from both groups. Both groups contained restriction fragments which hybridized to both 5' and 3' fragments of the cDNA clone, and it was concluded that the two groups were derived from two separate genes. Southern transfer analysis of individual chicken DNAs and quantitative hybridization analysis indicated that these two genes are independent and are present as single copies/haploid genome. Comparison of restriction digests of the cloned DNAs and total genomic DNA discounted the possibility that other closely related P-450 genes are present in the chicken genome.

The hemoprotein P-450 is the terminal enzyme of the pathway involved in the oxidation of a wide range of drugs and other xenobiotics. The extremely broad substrate specificity of P-450 is partly a result of the many different forms of the enzyme (1, 2) resulting from the multiplicity of genes in this multigene family. Generally, P-450s have been classified by the type of xenobiotic by which they are induced.

One of the most extensively studied P-450 families is that of the phenobarbital-inducible P-450s. In mammals, this family is complex and appears to contain many highly homologous genes. For example, several isozymes have been isolated from rat liver, each of which showed considerable polymorphism (3). Similarly, four isozymes of rabbit phenobarbital-inducible P-450 have been characterized either from sequencing of the isolated P-450 (4) or from cDNA clones (5). Filter hybridization analysis of mammalian genomic DNAs using cDNA clones specific for phenobarbital-inducible P-450s to detect related sequences revealed complex patterns of bands, suggesting the presence of at least six genes homologous to phenobarbital-inducible P-450b in the rat genome (9).

In view of the multiplicity of homologous P-450 genes in other species, we decided it was essential to determine the number of genes present in the chicken genome, prior to further work on the control of their expression. In this paper, we report the isolation and restriction mapping of a phage genomic clones representing a small family of phenobarbital-inducible P-450 genes. Several experimental approaches have been used to show that the family contains only two closely related genes.

EXPERIMENTAL PROCEDURES

Materials

Nitrocellulose (BA85), and DEAE-nitrocellulose (NA45) was from Schleicher & Schuell; restriction enzymes were from Pharmacia or New England Biolabs, and DNA polymerase, [α-32P]dATP, [α-32P]dCTP, [γ-32P]ATP, and synthetic oligonucleotides were from Bio-technology Research Enterprises S. A. Pty. Ltd., University of Adelaide, South Australia.

Methods

Library Screening and Preparation of Phage DNA—The HaeIII/Aul chicken genomic library in Charon 4A was kindly provided by Drs. J. Dodgson, J. Strommer, and J. Engel (10). The library was plated on Escherichia coli LES92 and screened according to the procedure of Maniatis et al. (11) using a cDNA clone, pCHP3, specific for a chicken phenobarbital-inducible P-450 (see accompanying paper (26)). After transfer of the phage to nitrocellulose, the filters were baked at 80 °C for 2 h and prehybridized at 52 °C in 6× SSC (1× SSC: 0.15 M NaCl, 0.15 M sodium citrate), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% SDS, and 200 μg/ml denatured sonicated salmon sperm DNA for a minimum of 4 h. Hybridizations were carried out for 36 h using the above conditions with 0.5–1×106 cpm/filter of [32P]labeled pCHP3 DNA. Filters were washed in 0.2× SSC, 0.1% SDS at 52 °C, air-dried, and exposed to x-ray film with an intensifying screen. All possible positive clones were picked and rescreened, giving plaque-pure preparations after four rounds of screening. Three clones required an extra screening to give plaque-pure preparations. Phage DNA was prepared using a minilysis procedure (11) with some modifications (12). One-tenth of each DNA preparation was digested with the appropriate restriction enzyme (2–5 units for 8–16 h) using the conditions recommended by the manufacturers.

Preparation of Total Chicken Liver DNA—Livers were removed from 8-week-old chickens and used immediately. Nuclei were prepared from individual livers in the presence of spermine, spermidine, EDTA, and EGTA according to the method of Marshall and Burgoyne (13). Total genomic DNA was carefully extracted using phenol and chloroform as described by Wallace et al. (14).

DNA Analysis—Labeled DNA probes were prepared by nick translation of cloned DNA molecules using both [α-32P]dATP and [α-32P]dCTP to give specific activities of 1–5×108 cpm/μg. Oligonucleotides were end-labeled using [γ-32P]ATP and polynucleotide kinase (11) to a specific activity of 1×1011 cpm/μg. The free nucleotides were separated from the labeled DNA using chromatography on Sephadex G-50.

DNA was digested with appropriate restriction enzymes, and the fragments were analyzed by electrophoresis on 0.8% agarose gels. The gels were stained with ethidium bromide, and the DNA fragments were transferred to nitrocellulose, either by the method of Southern

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; kb, kilobase; EGTA, ethylenediaminetetraacetic acid.
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RESULTS

Analysis of Total Genomic Chicken DNA—Prior to isolation of genomic clones, we analyzed total chicken DNA to assess the complexity of the phenobarbital-inducible P-450 gene family in chickens. Total chicken DNA was digested with EcoRI and BamHI, electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with nick-translated pCP3 at 68 °C (A) or 52 °C (B) as described under "Methods." No further specifically hybridizing restriction bands were detected.

Plasmid Subcloning and DNA Preparation—Prior to subcloning, restriction fragments were isolated using either of two methods. One technique involved separation of restriction fragments by electrophoresis on low melting point agarose followed by cutting out the necessary bands and isolating the DNA by phenol/chloroform extraction (11). The second involved electrophoresis of restriction digests on normal agarose gels. A DAE precursor cell line was then plated in front of the appropriate bands, and the DNA was electrophoresed directly onto the filter. The filter was rinsed to remove agarose, and the DNA was eluted in 1 M NaCl, 0.05 M arginine at 70 °C for 1 h. The eluate was centrifuged, the supernatant was extracted with phenol, and the DNA was precipitated with ethanol.

DNA fragments from both genomic and cDNA clones were subcloned into pBR322 (17). Vector DNA was cut with the appropriate restriction enzymes and purified on low melting point agarose gels. Ligation reactions were performed according to the method of Dagaiczky et al. (18) except that the molar ratio of vector to insert DNA was kept at 2:1 and ligations were performed at 17 °C for 4–6 h. Ligated plasmid DNAs were transformed into E. coli strain MC1061 (11) which was plated onto L plates supplemented with either 50 μg/ml ampicillin or 20 μg/ml tetracycline as required.

Initially, plasmid DNAs were prepared by the alkali minilysis extraction procedure of Birnboim and Doly (19) and analyzed by restriction enzyme digestion and agarose gel electrophoresis. Large-scale DNA preparation was carried out as described by Yu-Lee and Rosen (20).

Fig. 1. Hybridization analysis of total chicken DNA. Chicken DNA (10 μg) was digested with EcoRI (lanes 1) or EcoRI/BamHI (lanes 2) and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filters were probed with nick-translated pCP3 at 68 °C (A) or 52 °C (B) as described under "Methods." No further specifically hybridizing restriction bands were detected.

Isolation of Genomic Clones—About 1 × 106 phage from a chicken genomic library were screened with pCP3, and 29 recombinant phage were detected and plaque-purified. Restriction mapping of these genomic clones revealed that they were derived from two non-overlapping groups. One of these groups with 19 clones (group A; Fig. 2A) contained a 1.3-kb EcoRI fragment, a 3.8-kb EcoRI/BamHI fragment, and a 9.4-kb EcoRI fragment, each of which hybridized to the cDNA clone. Since total genomic chicken DNA does not contain a positively hybridizing 9.4-kb EcoRI fragment (Fig. 1), it was concluded the EcoRI site at the end of the insert in clone X3 was constructed during the cloning procedure. The second group, group B, contained a 1.3-, a 3.4-, and an 8.4-kb EcoRI/BamHI restriction fragment, each of which hybridized to pCP3. However, the EcoRI site binding the largest fragment was also constructed during cloning, and so the EcoRI 8.4-kb fragment represented a genomic fragment of indeterminate size. Clones X4, X8, and X10 also contained an extra BamHI site not present in other overlapping clones and probably represented a polymorphism.

Whether or not these two groups represented the two ends of the same gene was investigated using the three largest PstI fragments of pCP3H, subcloned into pBR322. The PstI insert in subclone pCP3A is 1.4 kb in length and contains a small part of the 5'-noncoding region as well as most of the coding region of the mRNA. pCP3B contains a 700-base pair insert covering a small part of the carboxyl-terminal half of the protein plus part of the 3'-noncoding region, whereas pCP3C contains only 3'-noncoding region (see accompanying paper (26)). These three subclones were used to analyze...
Fig. 2. Restriction maps of group A (A) and group B (B) genes. Two overlapping groups of genomic clones were distinguished after digestion with EcoRI and BamHI and Southern analysis using pCHP3 as probe. Restriction fragments from a few genomic clones as specified in the text were subcloned into pBR322 and mapped using the indicated restriction enzymes. Southern analysis of the restriction digests was performed using the cDNA subclones pCHP3A, pCHP3B, and pCHP3C. Solid and broken lines indicate strongly and weakly hybridizing bands, respectively. The restriction site marked with an asterisk is a BamHI site found only in the indicated clones. We suspect this represents an allelic variation.

EcoRI/BamHI digests of four clones covering the entire maps of groups A and B (Fig. 3). The results show that the subclone pCHP3A hybridized to all the fragments which hybridized to the entire cDNA clone. In group A, pCHP3A hybridized to the 1.3, and 9.4-kb EcoRI fragments and the 3.8-kb EcoRI/BamHI fragment (Fig. 3B, lanes 3 and 4). In group B, pCHP3A hybridized to the 1.3-, 3.4-, and 8.4-kb EcoRI/BamHI fragments (Fig. 3B, lanes 1 and 2). (pCHP3A also hybridized to a 2.7- and a 0.9-kb band, but these are only fragments of larger restriction enzyme fragments that have arisen as a result of the cloning procedure.) The subclone pCHP3C hybridized to both the 9.4-kb fragment of group A and the 8.4-kb fragment of group B (Fig. 3D). Subclone pCHP3B also hybridized to both of these larger bands as well as to the 3.4-kb EcoRI/BamHI fragment of group B (Fig. 3C). The reason for this latter anomalous hybridization is unknown but should be resolved by subsequent sequence analysis. Since all three regions of the cDNA clone were represented in both group A and B genes, it was concluded that both groups must represent separate genes.

To allow a better comparison of the two genes, selected restriction fragments were subcloned and detailed restriction analyses were performed. From group A, the 3.8-kb EcoRI/BamHI and the 1.3-kb EcoRI fragments of clone X3 and the 9.4-kb EcoRI fragment of clone X18 were isolated and subcloned into pBR322. Similarly, the 8.4-kb EcoRI/BamHI fragment of clone X20 and the 3.8-kb, 1.3-kb, and 2.7-kb EcoRI/BamHI fragments of clone X12 were subcloned in pBR322. Each subclone was subjected to detailed restriction analysis, and the restriction fragments were probed with the three subclones of pCHP3 (Fig. 2). The fine maps indicate that both genes are of a similar size (at least 12 kb), with pCHP3A, which contains most of the coding region, hybridizing to restriction fragments covering 10 and 7 kb of groups A and B, respectively. The maps also indicate that the coding region in both genes is interrupted by a number of introns and that the distribution of exons is probably different for the two genes. By contrast the subclones pCHP3B and pCHP3C hybridized to a small region close to the end of the gene maps. The anomalous hybridization of pCHP3B mentioned previously is limited to a short segment near the 5' end of the group B gene. Another anomaly is also apparent with the pattern of hybridization in both group A and B genomic clones. Sequence analysis of pCHP3 indicates a 5' to 3' order for the PstI fragments of pCHP3A, pCHP3B, and pCHP3C, respectively. But pCHP3C hybridized to a restriction fragment which mapped between fragments which themselves hybridized to pCHP3A and pCHP3B. We have proven the identity of the subclones used as probes using sequence analysis and have rigorously checked the restriction maps. At

Fig. 3. All three regions of the cDNA clone, pCHP3, hybridize to A and B genes. DNA from clones λ20 (lanes 1), λ12 (lanes 2), λ22 (lanes 3), and λ10 (lanes 4) was digested with EcoRI and BamHI and electrophoresed on a 0.8% agarose gel. A shows the ethidium bromide-stained gel. After transfer to nitrocellulose, the filters were probed with nick-translated pCHP3A (B), pCHP3B (C), or pCHP3C (D).
present, we do not know the reason for this spurious result, but isolation of further genomic clones from an independent library followed by sequence analysis should resolve this difficulty.

Whereas the detailed restriction maps (Fig. 2) indicated a large number of differences between the two genes, it was possible that these two maps represented two alleles from the same locus. In order to test this possibility, DNA was isolated from seven individual chicken livers and analyzed after digestion with EcoRI and EcoRI/BamHI (Fig. 4). All seven chickens contained the 1.3-kb fragment from group A (Fig. 4, A–G, lanes 1) and the 3.8- and 3.4-kb EcoRI/BamHI restriction fragments characteristic of groups A and B genes, respectively (Fig. 4, A–G, lanes 2). Assuming that the genes are alleles with equal frequency in the population, the probability of this happening is 1 in 128. If either allele were present at a lower frequency, then the probability would be even smaller. Therefore, it was concluded that the group A and B genes were probably not allelic polymorphisms but occur at separate loci in the chicken genome. In some of the EcoRI digests, a 4.8-kb band was visible, the presence of which correlated with the disappearance of a 20-kb band. In this case, we hypothesize that this band is due to allelic differences.

Further experiments were then carried out to determine whether there were other genes in the chicken genome homologous to pCHP3, clones for which had not been isolated from the genomic library. Therefore, chicken DNA along with DNA from four clones representing groups A and B were digested with EcoRI/BamHI (Fig. 5A), BamHI/SacI (Fig. 5B), and HindIII (Fig. 5C), electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with pCHP3. Digestion of the chicken DNA with EcoRI/BamHI gave four bands which hybridize to pCHP3. The 1.3-kb band was accounted for by 1.3-kb fragments in clones from both A and B genes. The 3.8- and 3.4-kb bands were accounted for by fragments in group A (lanes 2 and 4) and group B (lanes 3 and 5) genes, respectively. The largest band of about 15 kb must be due to fragments from both group A and B genes (lanes 2 and 3). The smaller size of the cloned fragments is due to removal of arms. This result is very strong evidence that there are only two genes (A and B) in the chicken genome which hybridize to pCHP3.

To determine the number of copies present in the genome of each of the two genes, A and B, quantitative hybridization analysis was carried out using the 3.8- and 3.4-kb EcoRI/BamHI restriction fragments of the genomic clones as characteristic markers for groups A and B, respectively. DNA isolated from a single chicken was digested with EcoRI and BamHI and run on two tracks of an agarose gel (Fig. 6, A and B, lanes 1). The 3.8- and 3.4-kb subclones were digested with the same enzymes, and amounts equivalent to 0.5, 1.0, 2.0, and 3.0 haploid genome equivalents were coelectrophoresed in adjacent lanes (Fig. 6, A and B, lanes 2–5, respectively). After transfer to nitrocellulose, the filter was hybridized with 32P-labeled pCHP3. Comparison of the strengths of the hybridization signals obtained from the genomic DNA with those obtained with the defined amounts of each subclone indicated that there was no more than one copy of either the 3.8- or the 3.4-kb restriction fragment/haploid genome. The extent of hybridization of pCHP3 to the 3.8-kb band from the genomic DNA was significantly less than that for the band in the lane containing one genome equivalent of plasmid DNA (A, lane 3). Hybridization to this band was found to be somewhat variable between experiments, but the results always indicated less than one copy. It was concluded that there was one group A and one group B gene/haploid genome.

Analysis of RNA from 2-allyl-2-isopropylacetamide- and 3,5-diethoxy carbonyl-1,4-dihydrocollidine-induced chick embryonic livers indicated the presence of two P-450 mRNA species of 2.5- and 3.5-kb in length. Further analysis of the RNA using synthetic oligonucleotides indicated at least one difference between the two mRNAs within their coding regions (see accompanying paper (26)). Two of these oligonucleotides were used to examine the relationship between the two mRNAs.

**Fig. 4.** Both A and B genes are present in all individual chicken genomes. DNAs (10 μg) from seven individual chicken livers (A–G) were digested with EcoRI (lanes 1) or EcoRI/BamHI (lanes 2). After transfer to nitrocellulose, the filters were probed with nick-translated pCHP3.
to the clones for the B gene at 42 °C. Raising the temperature to 52 °C had no effect on the binding to the group A clones but eliminated completely binding to the group B clones.

DISCUSSION

In this paper, we have reported the isolation and restriction mapping of genomic clones representing two genes of a phenobarbital-inducible P-450 gene family of chicken. The fine mapping results indicated that both genes were over 12 kb in length, and both contained at least five introns although the organization of the two genes is different, particularly in the region hybridizing to the 3' end of pCHP3A. This size and organization are consistent with the values of 1.4 kb and eight introns found for the rat phenobarbital-inducible P-450e (22). The rat P-450b gene is much larger, being 20 kb in length, but this extra size is due almost entirely to the first intron which is 12.5 kb in length compared to 3.2 kb in the rat P-450e gene (23). We are at present sequencing these chicken clones which will allow a far more detailed comparison between the chicken and mammalian genes. However, the results in Fig. 2 show a skewed distribution of the genomic clones with respect to the gene. Whereas many clones extend up to 10 kb past the 5’ end of the gene, a small region of the 3’ end of the gene may not be represented. Since the genomic library used in this study had been amplified at least twice, it is possible that the sequences representing the 3’ ends of these genes were selectively lost. Also, with the order of the restriction fragments which hybridize to pCHP3B and pCHP3C opposite to that expected, it is possible that some recombination events have occurred during the four or five rounds of screening that were used to isolate the clones. A second, independent library is currently being screened using a cDNA probe containing only the 3’ end of the mRNA which should allow resolution of these problems.

The comprehensive analysis presented here clearly indicates the presence of two independent phenobarbital-inducible P-450 genes in the chicken genome. Even very low stringency hybridization could not detect other genes of moderate homology to the A and B genes. A somewhat larger number of phenobarbital-inducible P-450 genes were detected in mammalian genomes using filter hybridization analysis (6–8), quantitative hybridization (9), and characterization of isolated genomic clones (7). However, hybridization analysis of rat genomic DNA using pCHP3 as the probe DNA only detected a few bands (results not shown), not the greater number of bands which have been reported to hybridize to the homologous rat P-450 cDNA (6, 7). Minor differences in the level of homology caused by slight differences in mutation rate could affect the detectability of specific genes by hybridization analysis. Therefore, there could be other distantly related phenobarbital-inducible P-450 genes in the chicken genome which were not detected in the present study.

It should be noted that membership of a particular P-450 gene family has been determined by homology with a cDNA clone derived from a drug-inducible mRNA. Thus, some members of these gene families may not necessarily be inducible by the appropriate drugs. For example, Atchison and Adesnik (7) concluded that only some of the genes in the rat phenobarbital-inducible gene family were in fact induced by phenobarbital. Also, rabbit P-450-3b can be considered to be a member of this family of P-450s based on sequence homology, but its expression is not greatly affected by phenobarbital (24).

The results of the hybridization analysis using the oligonucleotide probes indicate that at least gene A is active and is inducible by phenobarbital, giving rise to the 3.5-kb mRNA
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previously characterized (see accompanying paper (26)). At present, it is not possible to say whether gene B is actively transcribed to give the 2.5-kb mRNA or whether the 2.5- and 3.5-kb mRNAs are both derived from gene A, possibly by a differential splicing mechanism. We are currently performing further work including experiments involving transcription in isolated nuclei in an attempt to determine the activities of both of these agents.

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