Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8105–8108, December 1985
Genetics

Cloning and chromosomal location of the α- and β-globin genes from a marsupial

(*in situ* hybridization/fluorescence-activated cell sorter/genome evolution)

BRANDON WAINWRIGHT* AND RORY HOPE

Department of Genetics, University of Adelaide, South Australia, Australia 5001

Communicated by G. Pontecorvo, July 22, 1985

ABSTRACT **The cDNA sequences encoding the adult α- and β-globin polypeptides of a marsupial, the native cat *Dasyurus viverrinus*, have been cloned and their nucleotide sequences have been determined. Using these cDNA clones we have determined the chromosomal location of the native cat α- and β-globin genes by *in situ* hybridization to fixed metaphase chromosomes and by hybridization to DNA extracted from chromosomes purified by a fluorescence-activated cell sorter. Using two independent methods of gene assignment we have shown the α- and β-globin-like gene sequences to be asyntenic in the native cat, the α-globin-like sequences being on chromosome 2 and the β-globin-like sequences being on chromosome 4.**

Most gene mapping studies have concentrated on eutherian (placental) mammals. Much less is known about the other major group of mammals, the marsupials, which are thought to have diverged from placental mammals about 130 million years B.P. (1), a time point intermediate between the divergence of the avian/nonavian lines (270 million years B.P.) and the placental mammal radiation (85 million years B.P.) (2). Studies on the structure and arrangement of marsupial genes are clearly of importance because of the unique evolutionary position of marsupials.

Globin genes constitute perhaps the most extensively studied eukaryotic multigene family. Vertebrate hemoglobin molecules are composed of an association of four globin polypeptides, two α-like and two β-like chains. Recent molecular analysis of vertebrate globin genes has revealed many details of their organization (3). Homologies between the α- and β-globin genes indicate that they arose by duplication of a primordial globin gene about 560 million years ago (3). The α- and β-globin genes have been found to be closely linked in the amphibian *Xenopus laevis* (4) but are on separate chromosomes in the chicken and in all placental mammals studied to date (3, 5). The analysis of vertebrate globin genes has led to the identification of many basic evolutionary processes, such as gene duplication and gene conversion (6, 7). The marsupials are the only major vertebrate group in which there has not previously been a report of the nucleotide sequence of any structural gene. In view of the importance of studies on globin genes to our understanding of the processes involved in molecular evolution and the unique evolutionary position of the marsupials, we have cloned and sequenced the α- and β-globin genes in a marsupial, *Dasyurus viverrinus*, the native cat. By the use of two independent methods of gene assignment we have demonstrated that the α- and β-globin-like sequences are asyntenic in the native cat.

MATERIALS AND METHODS

Construction of a cDNA Library from Native Cat Reticulocytes. Anemia was induced by phenylhydrazine treatment and total reticulocyte RNA was extracted from four native cats. Total poly(A)-RNA was purified and a cDNA library was constructed by insertion of dC-tailed double stranded cDNA into the *Pst* I site of pBR322 as described by Wilson *et al.* (8). Putative globin cDNA clones were isolated by screening with cDNA prepared by using sucrose gradient-purified native cat reticulocyte mRNA as template. Hybrid-arrest translation studies were performed on selected clones by using the procedure of Paterson *et al.* (9).

Nucleotide Sequence Determination of Cloned Insert DNA. Restriction fragments were excised from 6% polyacrylamide gels and subcloned into bacteriophage M13 mp83 (10). The nucleotide sequence was determined by using the dideoxy sequencing technique with fractionation on 0.25-mm-thick 6% polyacrylamide gels (11). The order of each restriction fragment was determined by sequence comparison of overlapping fragments.

Preparation of Metaphase Chromosomes for *in Situ* Hybridization. A diploid cell line dv♂, established from a pinna explant from a male native cat, was cultured routinely in RPMI 1640 medium supplemented with 10% fetal calf serum. Chromosome preparations were obtained by treating logarithmically growing cultures with 0.02 μg of Colcemid per ml for 1 hr prior to harvesting. Standard air-dried slides were prepared and allowed to age for 2–3 days prior to hybridization. Slides were stored desiccated at −80°C for storage periods of >1 week.

Preparation of Labeled Hybridization Probes. Probes for *in situ* hybridization experiments were obtained by labeling linearized plasmid DNA by nick-translation with tritiated nucleotides to specific activities of 1–2 × 10^7 dpm (12). Probes for Southern blot analysis were labeled to specific activities of >2 × 10^8 cpm by nick-translation in the presence of α-^{32}P-labeled nucleotides.

***In Situ* Hybridization.** The *in situ* procedure used here was essentially that of Trent *et al.* (13). Slides were hybridized at probe concentrations ranging from 0.04 to 0.4 μg/ml in a 50% formamide solution containing 10% dextran sulfate and a final concentration of 250 μg of carrier DNA per ml. Slides were autoradiographed from 14 to 30 days at −80°C. Slides were stained in a 1% (wt/vol) solution of toluidine blue for 30 sec.

Flow Cytometry and Flow Sorting of Native Cat Chromosomes. Metaphase chromosomes for flow cytometric analysis were prepared as described (14). Ethidium bromide-stained chromosomes were analyzed and sorted at particle flow rates of up to 1000 particles per sec by using a Becton-Dickinson FACS IV at an excitation frequency of 488 nm. Chromosomes were sorted into chilled tubes, which were then

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*Present address: Department of Biochemistry, St. Mary's Hospital Medical School, Norfolk Place, Paddington, London W2 1PG, UK.

snap-frozen and stored at −80°C. Aliquots were routinely withdrawn and examined by analysis of Giemsa-stained chromosomes. Sorted chromosomes consisted of at least 80% of the chromosome type indicated in Fig. 5, with the main contaminant being random chromosomes and chromosome fragments, except for chromosome 1, in which the main contaminants were chromosomes 2 and 3.

Isolation and Analysis of DNA from Sorted Chromosomes. Each chromosome fraction consisting of ≈1 × 10^6 chromosomes of each type was thawed and pelleted by centrifugation at 50,000 × *g* for 3 hr at 4°C. Each aliquot was resuspended in 1.0 ml of digestion solution [200 μg of proteinase K per ml, 20 μg of sonicated, heat-denatured salmon sperm DNA per ml, 50 mM Tris·HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.5% $NaDodSO_4$]. After incubation at 45°C for 5 hr the mixture was extracted with phenol and precipitated by the addition of a 1/10 vol of 3 M sodium acetate (pH 5.5) and 2.5 vol of ethanol. After precipitation at −20°C for 72 hr the DNA was collected, resuspended in a final volume of 30 μl, and digested with 50 units of *Eco*RI for 3 hr at 37°C. Restriction digests were loaded directly onto 0.8% agarose gels, transferred to nitrocellulose membranes, and hybridized as described (12).

RESULTS

Screening of the Native Cat Reticulocyte Library. Recombinants were hybridized to 10S cDNA and positive clones were subjected to restriction enzyme analysis. Recombinants pDG 73 and pDG 5 were identified by hybrid-arrest translation as encoding the native cat adult α- and β-globin genes (results not shown).

Nucleotide Sequences of pDG 73 and pDG 5. Fig. 1 shows the complete sequence of the coding strand of the globin cDNA insert in pDG 73. Examination of the sequence reveals an open reading frame encoding a polypeptide of 141 amino acids. Comparison of the amino acid sequence encoded in pDG 73 with the published adult α-globin polypeptide sequence from the grey kangaroo and man (15) demonstrates that pDG 73 encodes the native cat α-globin polypeptide, showing 88% homology with kangaroo and 83% homology with the human amino acid sequences. Fig. 2 shows the complete sequence of the pDG 5 cDNA insert. An open reading frame of 258 nucleotides was found, which showed homology to the region of the adult human β-globin polypeptide (15) from amino acid 60 to the COOH terminus (81%) and to the same region of the adult grey kangaroo β-globin polypeptide (88%).

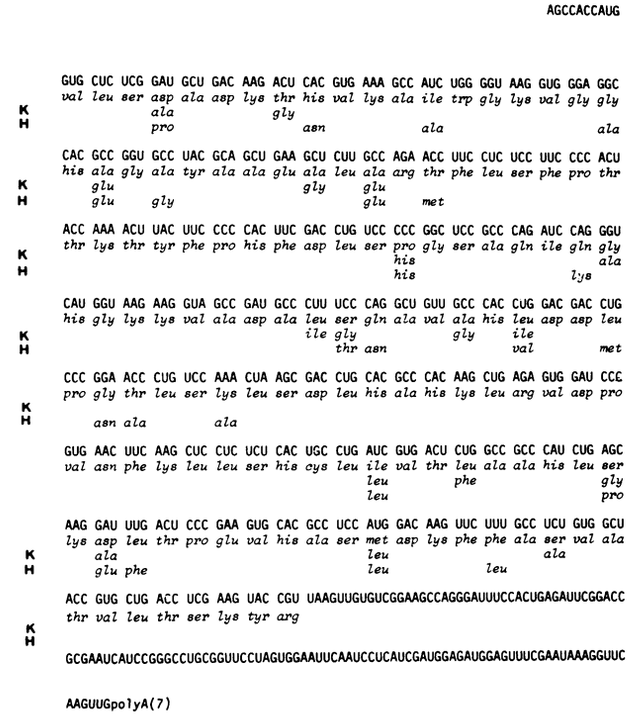

FIG. 1. Complete nucleotide sequence of the pDG 73 insert DNA. The inferred amino acid sequence is shown directly below. Amino acids that were found to differ from the grey kangaroo (K) and the human (H) α-globin amino acid sequence are indicated.

Localization of the α- and β-Globin Genes by *in Situ* Hybridization. A karyotype of the native cat derived from the cell line dv♂ is shown in Fig. 3. Metaphases hybridized to ^3H-labeled pDG 73 DNA (α-globin) at a concentration of 0.1 μg/ml were found to have an average of 1.3 labeled chromosomal sites per cell, with a total of 140 metaphases being analyzed. The results were tested for significance in the following manner. In the absence of significant hybridization of pDG 73 DNA to the native cat chromosomes, one would expect the silver grains to be distributed at random over all chromosomes. The expected number of grains per chromosome therefore is calculated by dividing the total number of grains present over chromosomes by the relative proportion of the genome represented by each chromosome. Fig. 4*A* shows the result of subtracting the expected values from those observed. The displacement from expected for chromosome 2 gave a *t* value of 2.15, which is significant at the 5% level ($0.05 > P > 0.025$). Chromosome 2 is a metacentric, and, in the absence of chromosome banding, it is not possible to unambiguously orientate each chromosome to determine the distribution of silver grains along its length. Therefore, chromosome 2 was divided by length into two classes, the central third and the telomeric thirds. The labeling of chromosome 2 with the pDG 73 probe was found to be regionally localized, with 65% of the grains mapping to the central third of the chromosome.

Similarly, after an exposure of 30 days to the pDG 5 probe (β-globin), an average of 1.4 labeled chromosomal sites per metaphase was observed, with a total of 70 metaphase spreads being analyzed. The data were analyzed in the same manner as for the pDG 73 probe (Fig. 4*B*). The displacement

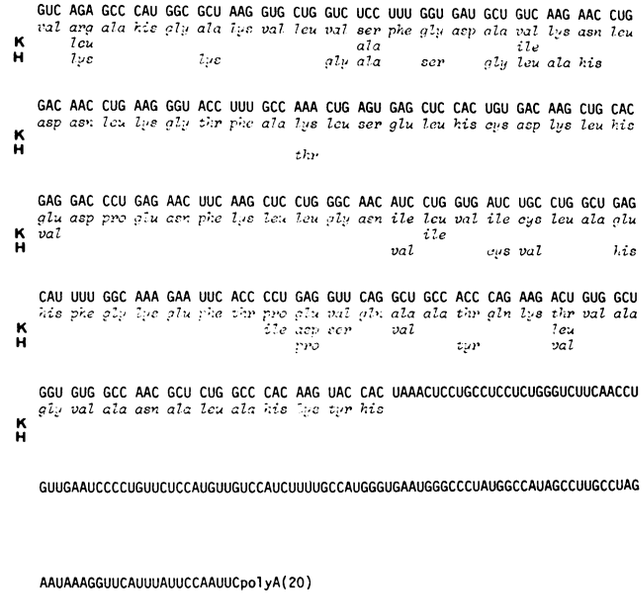

FIG. 2. Complete nucleotide sequence of the pDG 5 insert DNA. The inferred amino acid sequence is shown directly below. Amino acids that were found to differ from the grey kangaroo (K) and the human (H) β-globin amino acid sequence are indicated.

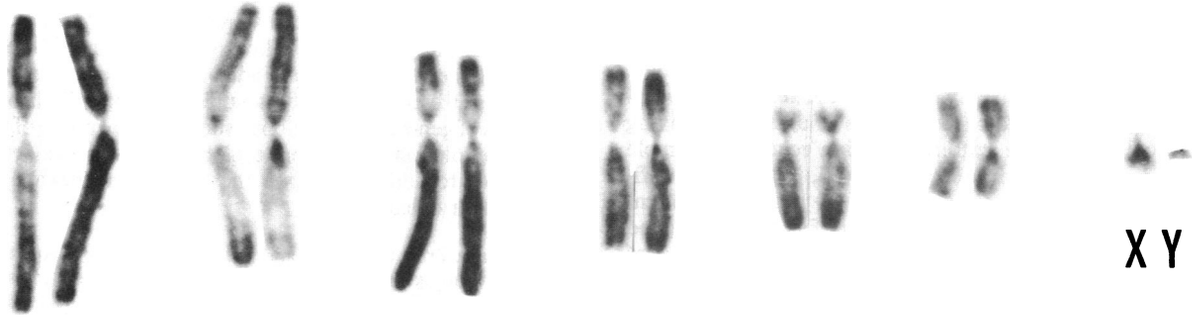

FIG. 3. Metaphase chromosomes of the male native cat. Autosomes are numbered 1–6 in order of decreasing size. The sex-determining chromosomes are indicated.

value observed for chromosome 4 gave a t value of 2.17, which is significant at the 5% level ($0.05 > P > 0.025$). Chromosome 4 was divided into thirds based upon chromosome length, and 76% of the labeled sites were found to reside within the central third.

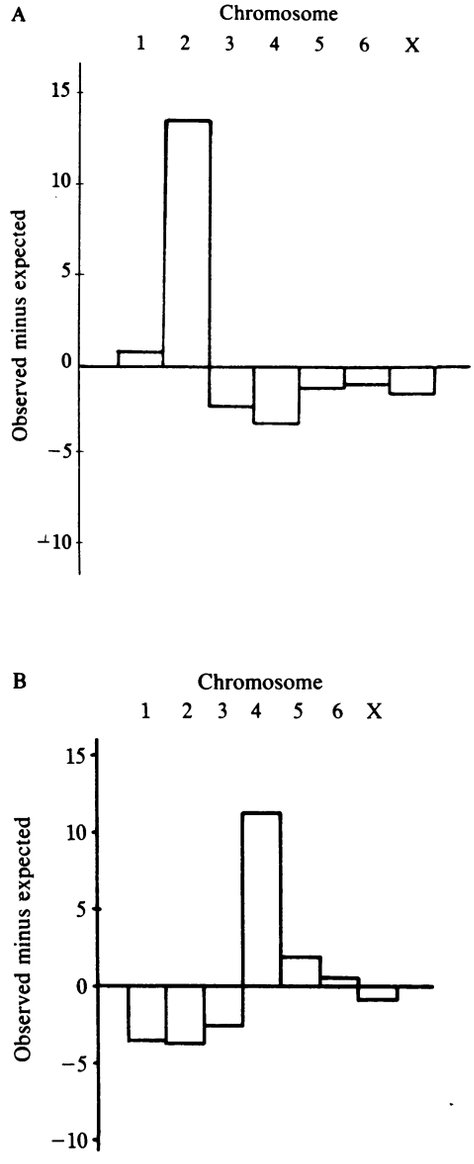

FIG. 4. Histogram showing the results of *in situ* hybridization experiments. (*A*) Hybridization of pDG 73 DNA (0.1 μg/ml) to native cat chromosomes. (*B*) Hybridization of pDG 5 DNA (0.2 μg/ml) to native cat chromosomes. The number of each chromosome is indicated.

Localization of Native Cat Globin Genes by Hybridization to DNA Extracted from Flow-Sorted Chromosomes. *α-Globin Gene sequences.* An initial sorting run was performed that separated the native cat chromosomes into two groups, one containing chromosomes 1–3 and the other containing the rest of the genome. At least 1×10^6 of each chromosome type was present in each sort. The flow sorting of native cat chromosomes has been described (14). The purity of each sort was monitored and each was found to consist predominantly of the chromosome types indicated in Fig. 5. After the sort containing chromosomes 1–3 was digested with *Eco*RI, specific hybridization to the pDG 73 probe could be detected. When chromosomes 1–3 were sorted into two groups, one containing chromosome 1 and the other chromosomes 2 and 3, hybridization of pDG 73 to the DNA from chromosomes 2 and 3 was observed, although a minor amount of hybridization could be seen to the sample consisting predominantly of chromosome 1 (Fig. 5). It was not possible to resolve chromosomes 2 and 3 by flow sorting (14).

β-Globin gene sequences. Native cat chromosomes were sorted into two groups, one group containing chromosomes 1–3 and the other containing chromosomes 4–6, X, and Y. Hybridization of plasmid pDG 5 to each sort resulted in the detection of β-globin-related sequences in the sort containing chromosomes 4–6, X, and Y (Fig. 5). Analysis of a sort containing only chromosome 4 demonstrated that all of the sequences hybridizing to the pDG 5 probe were found on that chromosome (Fig. 5).

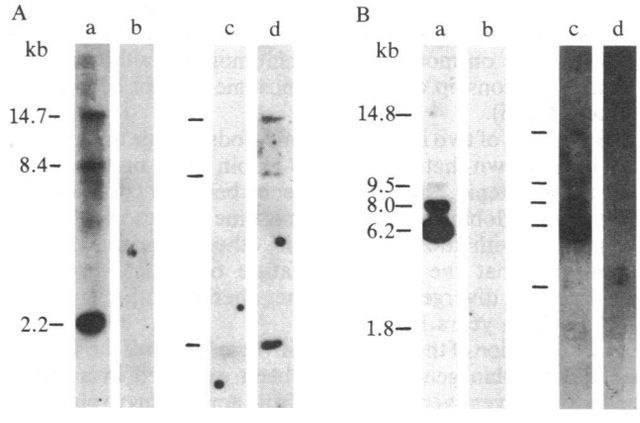

FIG. 5. (*A*) Hybridization of pDG 73 to *Eco*RI digests of DNA extracted from purified chromosomes 1–3 (lane a), 4–6, X, and Y (lane b), 1 (lane c), and 2 and 3 (lane d). All blots were washed to a stringency of 1.5 M NaCl. (*B*) Hybridization of pDG 5 to *Eco*RI digests of DNA extracted from purified chromosomes 1–3 (lane a), 4–6, X, and Y (lane b), 4 (lane c), 5, 6, X, and Y (lane d). All blots were washed to a stringency of 1.5 M NaCl. kb, Kilobases.

DISCUSSION

The native cat is a member of the super family Dasyuridae. Dasyurid marsupials display a highly conserved $2N = 14$ karyotype (16), with the chromosomes of all species having an identical G banding pattern or being related by simple inversions or reciprocal interchanges (16, 17). Due to difficulties encountered in the isolation of marsupial–rodent somatic cell hybrids (18, 19), the number of gene loci assigned in marsupials is low, with the chromosomal assignment of only three different X-linked loci and one autosomal locus having been confirmed (20, 21). No gene loci have been mapped previously in dasyurid marsupials. By using recombinant DNA techniques, the chromosomal location of any nucleotide sequences for which a suitable probe exists can be determined by analysis of somatic cell hybrids (22), *in situ* hybridization to fixed metaphase chromosomes (13, 23), or molecular analysis of purified metaphase chromosomes (24, 25). Accordingly, marsupial gene probes were isolated to facilitate the mapping of genes and obviate the need for a panel of marsupial/rodent hybrids.

We have isolated and determined the nucleotide sequence of cDNA encoding the major α- and β-globin polypeptides from the native cat *D. viverrinus*. The polypeptide sequences inferred from these cDNAs show a large degree of homology to the corresponding human and grey kangaroo sequences. To our knowledge, nucleotide sequences have not been reported previously for any marsupial gene. As shown in Figs. 1 and 2 the structure of the globin mRNA molecules in the native cat is not unlike that of other vertebrate globin mRNAs, with a 3′ noncoding region of comparable length to other globin mRNAs and the presence of a putative poly(A) signal motif AAUAAA in homologous positions within the molecule.

The results from the *in situ* hybridization experiments show specific localization of α- and β-globin-related sequences. After hybridization of the pDG 73 probe to samples of sorted chromosomes, some hybridization could be detected to the sample consisting predominantly of chromosome 1 and strong hybridization could be detected to the sample containing chromosomes 2 and 3. This was most likely due to a low level of contamination of the chromosome 1 sample with chromosomes 2 and 3 (14). An extensive genomic mapping study of native cat globin genes has not yet been performed. In the absence of such a study no conclusions can be reached as to which gene sequences are represented in Fig. 5. It is presumed that all of the hybridizing restriction fragments contain globin-like sequences, being major or minor, adult or fetal, or functional or pseudogenes. It is interesting to note that the related sequences do not appear to be on more than one chromosome, although their linkage relationship on that chromosome has not been determined (Fig. 5).

By the use of two independent methods of gene assignment we have shown that the α- and β-globin genes on the native cat are asyntenic, the α-globin genes being on chromosome 2 and the β-globin genes on chromosome 4. These results are consistent with those obtained in other mammals (3) and confirmed that the asyntenic nature of the globin genes predates the divergence of the metatherian/eutherian lines, ≈130 million years B.P.

The evolution of the chromosomal organization of the various α- and β-globin genes has now been examined in a representative of every vertebrate class from amphibians to placental mammals. It is generally held that early in vertebrate evolution multiple copies of a primordial α/β-globin locus were created and subsequent divergence and karyotypic rearrangement has resulted in the diversity gene arrangements observed between amphibians and mammals. The asyntenic nature of the α- and β-globin genes in marsupials adds further weight to this contention. Further analysis of marsupial globin genes through the analysis of genomic DNA clones should reveal much of interest on the evolution of vertebrate globin genes, a subject which continues to add insight into the forces and mechanisms of evolutionary change.

We thank Prof. G. Rogers and Dr. E. Kuczek for their assistance with the cloning procedures and for provision of facilities and Drs. J. Donald and S. O'Brien for their comments on the manuscript. B.J.W. was supported by a University of Adelaide postgraduate research studentship.

1. Air, G. M., Thompson, E. O. P., Richardson, B. J. & Sharman, G. B. (1971) *Nature (London)* **229,** 391–394.
2. Romero-Herrera, A. E., Lehmann, A. E., Joysey, K. A. & Friday, A. E. (1973) *Nature (London)* **246,** 389–395.
3. Jeffreys, A. J. (1981) in *Genetic Engineering 2*, ed. Williamson, R. (Academic, London), pp. 1–48.
4. Jeffreys, A. J., Wilson, V., Wood, D. & Simons, J. P. (1981) *Cell* **21,** 555–564.
5. Hughes, S. H., Stubblefield, E., Payvar, F., Engel, J. D., Dodgson, J. B., Spector, P., Cordell, B., Schimke, R. T. & Varmus, H. E. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1348–1352.
6. Liebhaber, S. A., Goossens, M. & Kan, Y.-W. (1981) *Nature (London)* **290,** 26–29.
7. Zimmer, E. A., Martin, S. L., Beverly, S. M., Kan, Y.-W. & Wilson, A. C. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2158–2162.
8. Wilson, J. T., de Riel, J. K., Forget, B. G., Marotta, C. M. & Weissman, S. M. (1977) *Nucleic Acids Res.* **4,** 2353–2358.
9. Paterson, B. M., Roberts, B. E. & Julf, E. L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4370–4374.
10. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9,** 309–321.
11. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1983) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
13. Trent, J. M., Celson, S. & Lawn, R. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7809–7813.
14. Wainwright, B. J. & Hope, R. M. (1984), *Aust. J. Biol. Sci.*, in press.
15. Dayhoff, M. O. (1975) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, DC).
16. Young, G. J., Marshall-Graves, J. A., Barbieri, J., Wolley, P. A., Cooper, D. W. & Westerman, M. (1982) in *Carnivorous Marsupials*, ed. Archer, M. (R. Zool. Soc. N.S.W., Sydney, Australia), pp. 56–62.
17. Rofe, R. (1979) Dissertation (University of Adelaide, Adelaide, Australia).
18. Hope, R. M. & Graves, J. A. M. (1978) *Aust. J. Biol. Sci.* **31,** 293–301.
19. Graves, J. A. M. & Hope, R. M. (1977) *Aust. J. Biol. Sci.* **30,** 461–469.
20. Donald, J. A. & Hope, R. M. (1981) *Cytogenet. Cell Genet.* **29,** 127–137.
21. Donald, J. A. & Adams, M. A. (1981) *Biochem. Genet.* **19,** 901–908.
22. Ouerbach, P., Rutter, W. J., Martial, J. A., Buxton, J. D. & Shows, T. B. (1980) *Science* **209,** 289–292.
23. Henderson, A. S. (1982) *Int. Rev. Cytol.* **76,** 1–43.
24. Lebo, R. V., Carrano, A. V., Burkhart-Schuttz, K., Doxy, A. M., Yu, L.-C. & Kan, Y. W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5804–5808.
25. Lebo, R. V., Kan, Y. W., Cheung, M. C., Carrano, A. V., Yu, L.-C., Chang, J. C., Cordell, B. & Goodman, H. M. (1982) *Hum. Genet.* **60,** 10–15.