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Genetics

Molecular definition of bovine argininosuccinate synthetase deficiency

(citrullinemia/animal model/nonsense mutation/polymerase chain reaction)

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ABSTRACT **Citrullinemia is an inborn error of metabolism due to deficiency of the urea cycle enzyme, argininosuccinate synthetase [L-citrulline:L-aspartate ligase (AMP-forming), EC 6.3.4.5]. The disease was first described in humans but was recently reported in dairy cattle in Australia. Here we report the nucleotide sequence of the normal bovine cDNA for argininosuccinate synthetase and the mutation present in animals with citrullinemia. Analysis of DNA from affected animals by Southern blotting did not readily identify the mutation in the bovine gene. RNA (Northern) blotting revealed a major reduction in the steady-state amount of mRNA in the liver of affected animals to <5% of controls. The bovine cDNA was cloned and sequenced and revealed 96% identity with the deduced human sequence at the amino acid level. Starting with mutant bovine liver, the mRNA was reverse-transcribed; the cDNA product was amplified with the polymerase chain reaction, cloned, and sequenced. The sequence revealed a C → T transition converting arginine-86 (CGA) to a nonsense codon (TGA). A second C → T transition represented a polymorphism in proline-175 (CCC → CCT). The mutation and the polymorphism were confirmed by amplification of genomic DNA and demonstration with restriction endonuclease enzymes of both the loss of an *Ava* II site in DNA from mutant animals at codon 86 and the presence or absence of a *Dde* I site at codon 175. The loss of the *Ava* II site can be used for rapid, economical, nonradioactive detection of heterozygotes for bovine citrullinemia.**

Argininosuccinate synthetase (L-citrulline:L-aspartate ligase (AMP-forming), EC 6.3.4.5) catalyzes the condensation of aspartate and citrulline into argininosuccinic acid with the concomitant hydrolysis of ATP to AMP and inorganic pyrophosphate. Its primary physiological role in ureagenic animals is in the liver, where it functions in the urea cycle. In humans, the disease associated with the inherited deficiency of the enzyme is citrullinemia (1, 2). This autosomal recessive disease is characterized by hyperammonemia due to the disruption in the urea cycle and usually results in coma and death if not treated vigorously.

The properties of argininosuccinate synthetase were initially delineated from protein obtained from bovine liver (3, 4). Argininosuccinate synthetase has since been studied extensively at both the protein and genetic levels, and reviews are available (5, 6). The enzyme has been purified from bovine (7), rat (8), and human liver (9). The cDNAs for human (10) and rat (11) have been sequenced, and the gene for the human locus has been described (12). The molecular basis for the disease in humans is very heterogeneous, with patients reported with partial enzyme activity (13), with and without immunoreactive protein (14), and with abnormal mRNA structure (15).

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Animal models for human diseases have been sought as they provide means for studying disease processes and for evaluating therapeutic strategies. However, not all animal models mimic human disorders. The mouse hypoxanthine phosphoribosyltransferase deficiency displays little clinical similarity to the devastating disease this enzyme deficiency causes in humans (16, 17). Harper *et al.* (18) have reported citrullinemia in the Friesian cattle breed (Friesian are a breed of dairy cattle identical to the Holstein breed commonly used in the United States) in Australia. The affected calves have a clinical disease that is similar to the acute neonatal form of the disease in humans. Other autosomal recessive disorders, including α-mannosidosis (19) and generalized glycogenosis (20) have been observed in cattle in Australia, and heterozygote detection tests have been developed and utilized to reduce the prevalence of the diseases (21, 22). To understand the molecular basis of the bovine argininosuccinate synthetase deficiency and perhaps to develop a heterozygote detection method, we undertook the investigation described herein and determined the sequence of normal bovine cDNA for argininosuccinate synthetase and the mutation present in animals with citrullinemia.‡

MATERIALS AND METHODS

Tissue Samples. Mononuclear cells were isolated from EDTA-treated blood by using a discontinuous Ficoll gradient (23). The cells were resuspended in 50 mM Tris·HCl (pH 7.5) containing 1 mM L-citrulline and frozen at −70°C until required for analyses. For establishment of skin fibroblast cultures, subcutaneous fibrous tissue was explanted and maintained in minimal essential medium (GIBCO) with 10% (vol/vol) fetal calf serum. Cells were harvested from 150-mm plates by scraping into 50 mM Tris·HCl (pH 7.5) containing 1 mM L-citrulline. The leukocytes and fibroblasts were frozen and thawed three times and centrifuged at 10,000 × *g* for 10 min, and the supernatant was analyzed for enzyme activity and protein.

Liver was collected from a normal animal and from an affected calf. Within 5 min of death, the tissue was frozen in liquid nitrogen and then held at −70°C until required for analyses.

Southern and Northern (RNA Blot) Analyses. DNA was extracted from leukocytes or cultured fibroblasts as described (24). RNA was extracted from liver by the method of Chirgwin *et al.* (25).

Southern blots were performed as described (24). The Nytran filters (Schleicher & Schuell) were probed with a full-length human argininosuccinate synthetase cDNA (26) prepared by hexamer labeling (27). After hybridization, the

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filters were washed for 5 min at room temperature with 2× SSC (1× SSC is 0.15 M NaCl/15 mM Na citrate, pH 7.0) containing 0.5% sodium dodecyl sulfate and then for 15 min at room temperature with 2× SSC/0.1% sodium dodecyl sulfate, followed by four washes each of 30 min at 67°C with 0.5× SSC/0.1% sodium dodecyl sulfate.

Northern blots were performed as described by Maniatis *et al.* (28). The nitrocellulose filters (Schleicher & Schuell) were probed with a human argininosuccinate synthetase cDNA and a human argininosuccinate lyase cDNA (29). The filters were washed according to the procedure described above.

Argininosuccinate synthetase and argininosuccinate lyase (EC 4.3.2.1) assays were performed as described (30, 31). Protein concentration in extracts was determined by using the BCA protein assay reagent (Pierce).

Cloning and Sequencing of Normal and Mutant Bovine cDNA. Bovine liver cDNA libraries were obtained from R. T. A. McGillivary (Department of Biochemistry, University of British Columbia, Vancouver) and D. T. Chuang (Department of Medicine, Veterans Administration Medical Center, Cleveland, OH). The libraries were screened with a human argininosuccinate synthetase cDNA probe following methods described by Maniatis *et al.* (28). Nitrocellulose filters were prehybridized and hybridized in the same manner as that described for probing Northern and Southern blots.

Nucleotide sequencing was determined by using the Sanger dideoxy method (32) with large-fragment DNA polymerase I (Boehringer Mannheim) or Sequenase (United States Biochemicals) (33) reactions followed by electrophoresis in gradient polyacrylamide gels (34).

RNA was extracted from the liver of an affected calf. Reverse transcription and amplification of cDNA were performed as described (35) by using a detailed protocol provided by that laboratory. Oligonucleotide primers for amplification of the entire coding region of the bovine cDNA were as follows: sense primer = 5′-(GCATGGATCC)CGTCACGATGTCCGGC-3′ and antisense primer = 5′-(CGTAGGATCC)GCATTGTCAGCTGGTCTA-3′. The primers included *Bam*HI linkers (shown in parentheses), and the product of the amplification reaction was cloned into pTZ18U for sequencing.

Direct Detection of Mutations by Amplification of Genomic DNA. Amplification of genomic bovine DNA was performed by using *Thermus aquaticus* (Taq) polymerase for 30–35 cycles of amplification as recommended by the supplier (Perkin–Elmer/Cetus). The primers within the exons were selected based on the knowledge of intron/exon boundaries in the human and mouse genes. The primers for amplification of the exon including codon 86 were as follows: sense primer = 5′-GTGTTCATTGAGGACATC-3′ and antisense primer = 5′-CCGTGAGACACATACTTG-3′. The primers for amplification of the exon containing codon 175 were as follows: sense primer = 5′-CAACATGGAATCCCCGTC-3′ and antisense primer = 5′-CTGATATGCATCAGGTTC-3′. Products of the amplification were digested with *Ava* I or *Dde* I under conditions recommended by the supplier (New England Biolabs or Boehringer Mannheim). Electrophoresis was performed either in 4% agarose or in 12% acrylamide gels, and products were visualized with ethidium bromide.

RESULTS

Biochemical Characterization. The diagnosis of citrullinemia in affected calves was established by analysis of citrulline levels in the serum as described (18). The diagnosis was confirmed by measurement of argininosuccinate synthetase activity in liver taken at autopsy (Table 1). Argininosuccinate synthetase activity in liver from affected calves was undetectable (<2% of control values). The activity for argininosuccinate lyase, the next enzyme in the urea cycle, was normal in the liver of affected animals. Activity of argininosuccinate synthetase was readily detectable in leukocytes of unaffected cattle. Although mean activity was lower in heterozygotes than in controls (Table 1), the ranges in the two groups overlapped substantially. Leukocytes were not available from affected animals. Enzyme activity was below the level of detection in cultured bovine fibroblasts. It is a biological curiosity that enzyme activity is undetectable in human leukocytes and easily measured in human fibroblasts, while the opposite is true for bovine samples. Enzymic analysis confirmed the diagnosis of citrullinemia in the calves and documented the biochemical and clinical similarity between the human and bovine disease.

Table 1. Activity of argininosuccinate synthetase in bovine extracts

Tissue	Mean activity, milliunits/mg of protein ± SD		
	Controls	Heterozygotes	Affected calves
Liver	17.60 ± 3.20 (5)	ND	<0.05 (6)
Leukocytes	0.40 ± 0.25 (21)	0.28 ± 0.10 (19)	ND
Fibroblasts	<0.05 (6)	<0.05 (3)	<0.05 (2)

The number of animals is in parentheses. ND, not determined.

Southern Blot Analysis. In order to assess the possibility that a major rearrangement in the gene accounted for the loss of enzyme activity, Southern blot analysis was performed with human argininosuccinate synthetase cDNA as a probe. Analysis with 35 different restriction enzymes comparing affected, obligate heterozygote, and presumed homozygous normal animals failed to identify alterations that might be consistent with the presence or absence of the mutation (data not shown). Numerous restriction fragment length polymorphisms were identified when Friesian DNA was compared with DNA from animals with some non-Friesian ancestry. No polymorphisms were identified which would be useful for genetic diagnosis within the Friesian population. The enzymes *Ava* II and *Dde* I failed to readily detect aberrant patterns, which is significant given the results to be described below.

Northern Blot Analysis. RNA was isolated from the liver of normal and diseased animals. With the human argininosuccinate synthetase cDNA as a probe, a substantial reduction of mRNA was documented in the diseased (Fig. 1, lanes D) compared with the normal (Fig. 1, lanes N) animal. The amount of mRNA in the diseased animal was <5% of that in

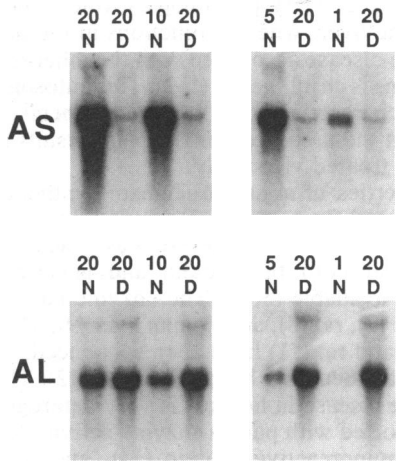

FIG. 1. Northern blot analysis of bovine liver. The amounts of total RNA loaded are indicated in micrograms for normal (lanes N) and diseased (lanes D) animals. Duplicate filters were hybridized with human argininosuccinate synthetase (AS) cDNA (*Upper*) or with human argininosuccinate lyase (AL) (*Lower*) as probe.

the normal animal (Fig. 1). The quality of the RNA from the affected animal was established by analysis with the human argininosuccinate lyase cDNA as a probe.

Isolation and Sequencing of the Bovine cDNA. As a step towards defining the molecular basis of the mutation, we screened cDNA libraries prepared from bovine livers by using the human cDNA as a probe. The sources of the livers used in making the cDNA libraries are not known. Multiple clones from two independent libraries were selected and sequenced. Fig. 2 shows the complete nucleotide sequence of the bovine cDNA. A comparison of the bovine sequence with the human sequence (10) revealed 103 base differences within the coding region, with 85 (81%) occurring in the third position of the codon. The base differences resulted in 16 amino acid changes (10 of these were conservative) in the bovine sequence compared with the human protein sequence, indicating 96% identity (98% with conservative changes). The predicted amino acid composition of the

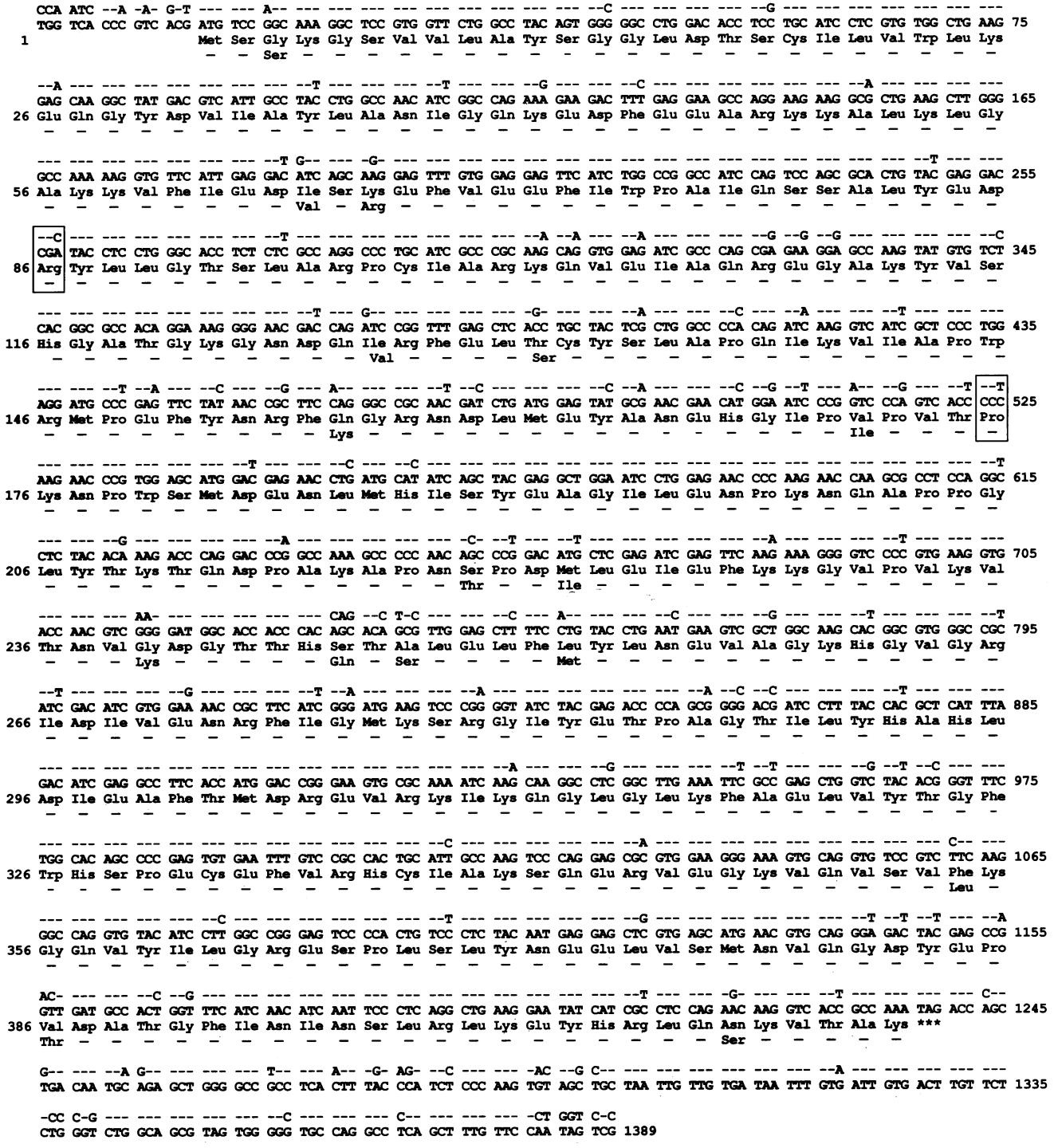

FIG. 2. The cDNA and deduced amino acid sequence for bovine argininosuccinate synthetase. For comparison, the nucleotide sequence of the human cDNA is depicted above and the deduced amino acid sequence of the human enzyme is below the bovine sequence. Dashes in the human sequence represent nucleotide identity with the bovine sequence. Numbers to the left of the figure refer to amino acid residues, and numbers to the right refer to nucleotides. The nucleotides are numbered with the adenosine of the initiation codon at +1. The sites of the nucleotide variations in the mutant sequence are enclosed in boxes. During the course of this work, K. Kobayashi in our laboratory discovered errors in the sequence of the cDNA for human argininosuccinate synthetase. These involved bases 975 to 981 and are shown correctly in this figure.

Table 2. Details of the mutations detected in bovine citrullinemia

Normal	Mutant	Result
	Mutation 1	
Nucleotide sequence		
GAG GAC ĊGA TAC CTC	GAG GAC ṪGA TAC CTC	C → T transition; loss of *Ava* II site
Amino acid sequence		
E D R Y L	E D * Y L	Arginine → nonsense; termination at amino acid residue 86
	Mutation 2	
Nucleotide sequence		
GTC ACC CCĊ AAG AAC	GTC ACC CCṪ AAG AAC	C → T transition; gain of *Dde* I site
Amino acid sequence		
V T P K N	V T P K N	No change

The site of nucleotide alteration in the sequence is denoted by a dot above the base. The position of the restriction site change is underlined. The asterisk indicates a termination codon.

protein differs little from the composition determined by Ratner (36) from the purified bovine protein.

Identification of the Bovine Mutation. Total mRNA was prepared from the liver of an affected animal, and cDNA was synthesized by using reverse transcriptase. The product was amplified by using the polymerase chain reaction. Two clones were selected from each of two independent amplification reactions, and four clones were sequenced in their entirety. Although occasional single-base differences were found in individual clones, only two single-base changes occurred in all of the clones as compared with the normal sequence (Table 2). The first base change is a C → T transition in the first position of codon 86 (counting the initiator methionine as codon 1) of the bovine protein. This change converts the CGA arginine codon to a TGA termination codon. This nonsense mutation should cause complete loss of enzyme activity. The mutation would predict the loss of an *Ava* II site in the genomic sequence. Assuming the intron/exon boundaries of the human and mouse genes are similar to the bovine boundaries, this base change should occur in the midportion of the fifth exon of the gene.

The second consistent difference between the mutant and the normal sequence also involved a C → T transition; this change was in the third position of codon 175 (CCC to CCT). This difference would represent a silent mutation with no amino acid alteration, but the difference should result in a gain of a *Dde* I site in DNA from animals with this change (Table 2). Even in retrospect, the restriction fragment length differences predicted by these mutations could not be recognized on Southern blot analysis apparently because the fragments were small and were only faintly visualized.

A method to confirm the presence of the mutations in genomic DNA and to analyze numerous animals for the mutations was sought. Although bovine genomic DNA sequence was not available, both mutations occurred within the midportions of exons, and it was feasible to amplify exon sequences by using the polymerase chain reaction. The results demonstrating the loss of the *Ava* II site in the case of the nonsense mutation are presented in Fig. 3. These data confirm that the predicted base change is present in the affected animals in the structural gene and is not an artifact of the amplification procedure. The base change in codon 175 was also confirmed to result in the gain of a *Dde* I restriction enzyme site. The presence of the *Dde* I site was found in homozygous form in some obligate heterozygotes and was found in heterozygous form in some animals who were homozygous for the presence of the *Ava* II site—i.e., in noncarriers. We conclude from these data that the base change resulting in the *Dde* I site is a normal polymorphism in Australian Friesian cattle. Due to the small number of animals analyzed for the *Dde* I polymorphism, we are unable to establish allele frequencies for this polymorphism.

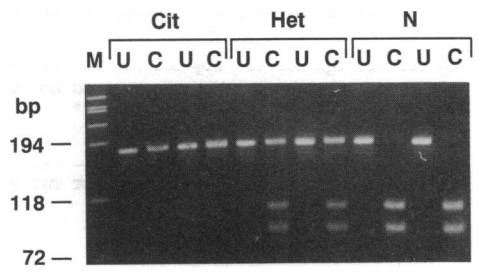

FIG. 3. Amplification of genomic DNA for detection of the bovine nonsense mutation. DNA (0.5 μg) was amplified and was analyzed without [lanes U (uncut)] and with [lanes C (cut)] digestion with *Ava* II from affected animals (lanes Cit), normal animals (lanes N), and obligate heterozygotes (lanes Het).

DISCUSSION

Two single-base differences between the mutant and normal bovine cDNA sequence for argininosuccinate synthetase were identified. Presumably the nonsense mutation results in the loss of enzyme activity, while the other nucleotide difference represents a DNA polymorphism. Nonsense mutations frequently result in a major reduction in the steady-state level of mRNA (37, 38), and we do not believe that any other mutation need be invoked to explain the low levels of mRNA in the mutant liver.

Bovine citrullinemia appears to be clinically and biochemically similar to the human disease. The animal model presents opportunities for study of pathogenesis and for evaluation of therapeutic intervention. The animal model may be useful for evaluating organ transplantation and somatic gene therapy for treatment of citrullinemia. Attempts are being undertaken to maintain affected animals by using dietary and pharmacologic therapy similar to that used in humans (2).

The ability to combine the polymerase chain reaction for amplification of DNA with restriction enzyme digestion of the product provides a simple and economical method for heterozygote detection for this form of bovine citrullinemia. The mutation is widespread in the Australian Friesian population, and the ability to unequivocally identify heterozygotes should permit efficient reduction of the prevalence of citrullinemia in this population.

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1. Beaudet, A. L., O'Brien, W. E., Bock, H.-G. O., Freytag, S. O. & Su, T.-S. (1986) *Adv. Hum. Genet.* **15,** 161–196.
2. Brusilow, S. W. & Horwich, A. L. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), pp. 629–663.
3. Ratner, S. & Petrack, P. (1953) *J. Biol. Chem.* **200,** 161–174.
4. Petrack, B. & Ratner, S. (1958) *J. Biol. Chem.* **233,** 1495–1500.
5. Jackson, M. J., Beaudet, A. L. & O'Brien, W. E. (1986) *Annu. Rev. Genet.* **20,** 431–464.
6. Ratner, S. (1973) *Adv. Enzymol.* **39,** 1–90.
7. Rochovansky, O., Kodowaki, H. & Ratner, S. (1977) *J. Biol. Chem.* **252,** 5287–5294.
8. Saheki, T., Kusumi, T., Takada, S. & Katsunuma, T. (1977) *J. Biochem. (Tokyo)* **81,** 687–696.
9. O'Brien, W. E. (1979) *Biochemistry* **18,** 5353–5356.
10. Bock, H.-G. O., Su, T.-S., O'Brien, W. E. & Beaudet, A. L. (1983) *Nucleic Acids Res.* **11,** 6505–6512.
11. Surh, L. C., Morris, S. M., O'Brien, W. E. & Beaudet, A. L. (1988) *Nucleic Acids Res.* **16,** 9352.
12. Freytag, S. O., Beaudet, A. L., Bock, H.-G. O. & O'Brien, W. E. (1984) *Mol. Cell. Biol.* **4,** 1978–1984.
13. Saheki, T., Nakano, K., Kobayashi, K., Imamura, Y., Itakura, Y., Sase, M., Hagihara, S. & Matuo, S. (1985) *J. Inherited Metab. Dis.* **8,** 155–156.
14. Su, T.-S., Bock, H.-G. O., Beaudet, A. L. & O'Brien, W. E. (1982) *J. Clin. Invest.* **70,** 1334–1339.
15. Su, T.-S., Beaudet, A. L. & O'Brien, W. E. (1983) *Nature (London)* **301,** 533–534.
16. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326,** 292–295.
17. Kuehn, M. R., Bradley, A., Robertson, E. J. & Evans, M. J. (1987) *Nature (London)* **326,** 295–298.
18. Harper, P. A. W., Healy, P. J., Dennis, J. A., O'Brien, J. J. & Rayward, D. H. (1986) *Aust. Vet. J.* **63,** 378–379.
19. Hocking, J. D., Jolly, R. D. & Batt, R. D. (1972) *Biochem. J.* **128,** 69–73.
20. O'Sullivan, B. M., Healy, P. J., Fraser, I. R., Nieper, R. E., Whittle, R. J. & Sewell, C. A. (1981) *Aust. Vet. J.* **57,** 227–229.
21. Healy, P. J., Babidge, P. J., Embury, D. H., Harrison, M. A., Judson, G. J., Mason, R. W., Petterson, D. S. & Sinclair, A. J. (1983) *Aust. Vet. J.* **60,** 135–136.
22. Healy, P. J. (1982) *Biochem. Med.* **28,** 224–228.
23. Healy, P. J. (1982) *Res. Vet. Sci.* **33,** 275–279.
24. Zoghbi, H. Y., Daiger, S. P., McCall, A., O'Brien, W. E. & Beaudet, A. L. (1988) *Am. J. Hum. Genet.* **42,** 877–883.
25. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
26. Wood, P. A., Partridge, C. A., O'Brien, W. E. & Beaudet, A. L. (1986) *Somatic Cell. Mol. Genet.* **12,** 493–500.
27. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
28. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
29. O'Brien, W. E., McInnes, R., Kalumuck, K. & Adcock, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7211–7215.
30. Su, T.-S., Beaudet, A. L. & O'Brien, W. E. (1981) *Biochemistry* **20,** 2956–2960.
31. O'Brien, W. E. & Barr, R. H. (1981) *Biochemistry* **20,** 2056–2060.
32. Sanger, F. (1981) *Science* **214,** 1205–1210.
33. Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4767–4771.
34. Biggin, M. D., Gibson, T. J. & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3963–3965.
35. Todd, J. A., Bell, J. I. & McDevitt, H. O. (1987) *Nature (London)* **329,** 599–604.
36. Ratner, S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5197–5199.
37. Daar, I. O. & Maquat, L. E. (1988) *Mol. Cell. Biol.* **8,** 802–813.
38. Atweh, G. F., Brickner, H. E., Zhu, X. X., Kazazian, H. G. & Forget, B. G. (1989) *J. Clin. Invest.* **82,** 557–561.