AN ELECTROPHORETICALLY SILENT POLYMORPHISM FOR THE BETA CHAINS OF RABBIT HEMOGLOBIN AND ASSOCIATED POLYRIBOSOME PATTERNS[1]

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ABSTRACT

The β chain of rabbit (*Oryctolagus caniculus*) hemoglobin has previously been reported to contain a single residue of isoleucine at β^{112}. We have detected other rabbits with either zero isoleucyl residues or half a residue per β chain. This character is polymorphic and inherited as a simple mendelian autosomal codominant.—Normally the modal number of ribosomes per polyribosome is 4 to 6 in reticulocyte lysates; but incubation of rabbit reticulocytes prior to lysis with L-o-methylthreonine (OMT), an isostere of isoleucine, leads to a bimodal distribution in lysates with 2–3 and 8–12 ribosomes as modes. This alteration has been attributed to ribosomal traffic jams caused by starvation for ile-tRNA at mRNA codons corresponding to the locations of isoleucyl residues at positions α^{10}, α^{17}, α^{55} and β^{112}. We have confirmed this interpretation by incubating OMT with reticulocytes from rabbits with integral, half integral and nil values for isoleucyl residues per β chain to show that formation of the larger clusters of polyribosomes requires that $\beta^{112} =$ ile.

NEARLY all polymorphisms in proteins have been detected in surveys using electrophoresis (*c.f.*, HARRIS 1966; HUBBY and LEWONTIN 1966; LEWONTIN and HUBBY 1966), a technique which may miss about two-thirds of all mutations involving amino acid substitutions (HARRIS 1971). BOYER (1972) has pointed out that electrophoretically silent polymorphisms can be detected via amino acid composition analysis of tryptic peptides. Similar polymorphisms, also not detectable by electrophoresis, have been found previously for the α (VON EHRENSTEIN 1966) and β (GALIZZI 1971) chains of rabbit hemoglobin. In this report we show that the presence or absence of isoleucyl residues in the β globin chain is a polymorphism in laboratory rabbits. Surveys using starch gel (BOYER, FAINER and NAUGHTON 1963) and disc (ORNSTEIN and DAVIS 1964) electrophoresis turned up only one rabbit with a hemoglobin variant in over 2,000 animals (GARRICK and SAUNDERS, unpublished). This lack of electrophoretic variants strongly suggests that, when isoleucine is absent, another neutral amino acid replaces it. Indeed, recently β^{112} ile $\rightarrow$ val has been identified as the substitution (BRICKER and GARRICK 1973; HONIG, personal communication).

Typically, isoleucine represents from 3% to 8% of the amino acid composition of a given protein; however, isoleucyl residues are rare or absent in the hemoglobins of eutherian mammals (DAYHOFF 1972). In rabbit hemoglobin, for example, isoleucine is present only at residues 10, 17 and 55 of the 141 amino acids in the α chain (VON EHRENSTEIN 1966; BRAUNITZER et al. 1968) and residue 112 of the 146 in the β chain (BRAUNITZER et al. 1966; BEST, FLAMM and BRAUNITZER 1969; GALIZZI 1970). Six years ago, studies of isoleucine incorporation into rabbit hemoglobin led us to suspect that some rabbits had half an isoleucyl residue, and others, none per β chain. We now show that the substitution at β^{112} is inherited as a simple mendelian codominant trait. We also demonstrate that an experimentally inducible alteration of the reticulocyte polyribosome pattern is heritable and requires that residue β^{112} be isoleucine, not valine.

When reticulocytes are incubated in an amino acid mixture with L-o-methylthreonine (OMT) replacing isoleucine, isoleucyl-tRNA becomes the limiting factor for protein synthesis (SMULSON and RABINOVITZ 1968) since OMT is adenylated but not ligated to tRNA by the ile-tRNA ligase. After rabbit reticulocytes are incubated with OMT, the polyribosome pattern in lysates is bimodal with 2 to 3 ribosomes per cluster and 8 to 12 ribosomes per cluster as modes (HORI and RABINOVITZ 1968). These results have been interpreted as representing ribosomal traffic jams at the isoleucyl codons near the beginning of the α mRNA and the end of the β mRNA, respectively. We demonstrate that the presence of isoleucine at β^{112} is necessary for the appearance of clusters containing more than 7 ribosomes when ile-tRNA is limiting.

MATERIALS AND METHODS

Rabbits: Domestic rabbits (*Oryctolagus caniculus*) were purchased from two local suppliers. Most were of the New Zealand White variety, although several were Flemish Giants or hybrids of the two. Heparinized blood was collected by arteriopuncture, venesection and cardiac puncture.

Determining amino acid content of β chains: Erythrocytes were washed 3× with 0.85% (w/v) NaCl by centrifuging at 1000 × g for 10 min at 4°, then lysed with distilled H_2O and the stroma removed by centrifuging at 18,000 × g for 10 min at 4°. Globin was prepared from hemolysates as described (GARRICK, DEMBURE and GUTHRIE 1973) and resolved into α and β chains by chromatography on carboxymethylcellulose (DINTZIS 1961) with a formic acid-pyridine buffer gradient (RABINOVITZ and FISHER 1964). The β chain fractions were pooled and dry protein was collected either by diluting 5× with distilled H_2O (to prevent losses) then freeze-drying or by precipitating through the addition of 50% (w/v) CCl_3COOH to yield a final concentration of 10% then collecting the precipitate on a glass fiber filter (Whatman GF-A) and washing it free of CCl_3COOH first with diethyl ether/95% ethanol (1:1) then with diethyl ether. The β chains were then hydrolyzed in 6N HCl for 72 hours at 110° under N_2. The hydrolysate was dried *in vacuo* and the composition determined on a Beckman 120B Amino Acid Analyzer with a Durrum single column buffer system.

Reticulocyte induction and handling: Rabbits were made anemic by phenylhydrazine injection (BORSOOK et al. 1952). Heparinized reticulocyte-rich whole blood was incubated with 25 $\mu c/ml$ of ^3H-L-isoleucine (New England Nuclear Corp.) or 10 $\mu c/ml$ ^{14}C-amino acids (Schwarz/Mann) at 37° for 4 hours under 95% $O_2/5\%$ CO_2 to determine incorporation into α and β chains. After the incubation, hemolysates were prepared, then resolved into globin chains as described above. The absorbance at 280 nm was determined for each fraction and incorporation was measured by liquid scintillation counting of an 0.5 ml aliquot dissolved in 6 ml of Econosol (Isolab, Inc.).

For analysis of polyribosomes, reticulocytes were washed 2× with NKM (140 mM NaCl, 5 mM KCl, 1.5 mM MgCl$_2$—WARNER *and* RICH 1964) and 1× with the incubation medium (below) at 1000 × g for 10 minutes at 4°. The medium contained amino acids at concentrations optimal for hemoglobin synthesis (BORSOOK, FISCHER and KEIGHLEY 1957) in NKM with 0.2% (w/v) dextrose, 29 μM penicillin G, 12 μM streptomycin, 13 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 10 mM Tris-(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5. Reticulocytes plus the above medium were incubated at 37° under 95% O$_2$/5% CO$_2$ for 15 or 30 min. Incubations were terminated by adding ice-cold NKM, the cells washed 2× with NKM, then lysed with eight volumes of 0.1% (w/v) saponin in TKM (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 1.5 mM MgCl$_2$) and the stroma removed by centrifuging at 18,000 × g for 10 min at 4°. One ml of each hemolysate was gently layered on 38 ml of a 15–50% (w/v) linear sucrose gradient in TKM. The gradients were centrifuged at 91,000 × g for 4 hours at 5° in a Beckman SW-27 rotor on a Beckman L2–50 ultracentrifuge. The gradient tubes were punctured at the bottom and the absorbance at 260 nm was monitored by pumping through a flow cell.

RESULTS

Table 1 shows typical values for the amino acid composition of the β chains of rabbit hemoglobin. The values for isoleucyl residues suggest three phenotypic classes which we have designated *1 Ile, 0.5 Ile* and *0 Ile*. Figure 1 summarizes results from a series of rabbits using β chain hydrolysates as the initial criterion. The distribution is clearly trimodal with modes at 0, 0.5 and 1 residue of isoleucine. For several rabbits, however, the initial data are intermediate between two modes. The difficulty in classifying them is attributable to two competing technical problems: incomplete hydrolysis which lowers the recovery of isoleucyl residues and contamination of β by α chains which raises the recovery. To determine the number of isoleucyl residues per β chain for rabbits with borderline values (0.15–0.35 or 0.65–0.85) and to verify the data for others, incorporation of ^{3}H-isoleucine into β chains was determined as shown in Figure 2. Isoleucine is

TABLE 1

Amino acid compositions

	Rabbit with			Nearest integer
Residue*	1 Ile	0.5 Ile	0 Ile	
Asx	12.98	11.80	12.39	12
Glx	14.88	13.51	14.81	14
Gly	10.37	12.90	10.68	11
Ala	14.30	15.45	14.97	15
Val	17.52	18.42	18.61	18
Met	0.93	1.01	1.08	1
Ile	0.87	0.58	0.06	1,0.5,0
Leu	18.17	17.00	17.48	18
Tyr	2.88	3.03	2.04	3
Phe	8.20	7.36	7.70	8
Lys	12.42	10.69	11.86	12
His	9.49	9.04	8.89	9
Arg	2.76	3.21	2.77	3

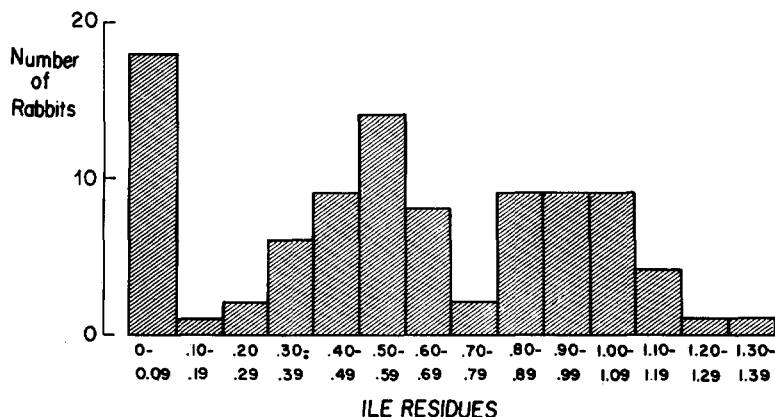* Thr, Ser, Trp and Cys omitted due to losses during hydrolysis; Pro, due to poor quantitation.

FIGURE 1.—Initial screening values for isoleucyl residues per β chain in laboratory rabbits. The quantity of isoleucine was calculated for hydrolysates of purified β chains assuming that each of the remaining twelve types of amino acids listed in Table 1 was present at the integer value also shown in Table 1.

not incorporated into the β chain region when the rabbit is the *0 Ile* type, while the α/β incorporation ratio closely approximates the expected values of 6 and 3 for the *0.5 Ile* and the *1 Ile* types, respectively. Incorporation data allowed us to classify rabbits as *0, 0.5* or *1 Ile* when the amino acid composition data were ambiguous and confirmed 13 of 14 initial assignments which had been considered unambiguous.

The simplest genetic interpretation of the foregoing is mendelian codominant inheritance. Table 2 summarizes population data on this polymorphism. The close agreement with Hardy-Weinberg expectation supports this interpretation, the slight deficit in heterozygotes being attributable to sampling two populations and/or to nonrandom breeding structure. Table 3 summarizes mating data. These confirm that the *1 Ile* and *0 Ile* types are homozygotes and *0.5 Ile* represents the heterozygote. To denote genotypes, we therefore use the residues at β^{112} with the homozygotes, *ile/ile* and *val/val*, and heterozygote, *ile/val*.

In Figure 3, reticulocyte polyribosome patterns are depicted. With optimal amino acid concentrations, patterns from the three genotypes are indistinguishable. Five ribosomes per cluster is the most populous class, and 4 and 6 per cluster next (Figure 3A–C). When 25 mM OMT replaces isoleucine in the amino acid

TABLE 2

Population data

Type	1 Ile	0.5 Ile	0 Ile
Observed	25	31	19
Expected*†	22	37	16

* Based on the Hardy-Weinberg equilibrium with p=0.54 and q=0.46.
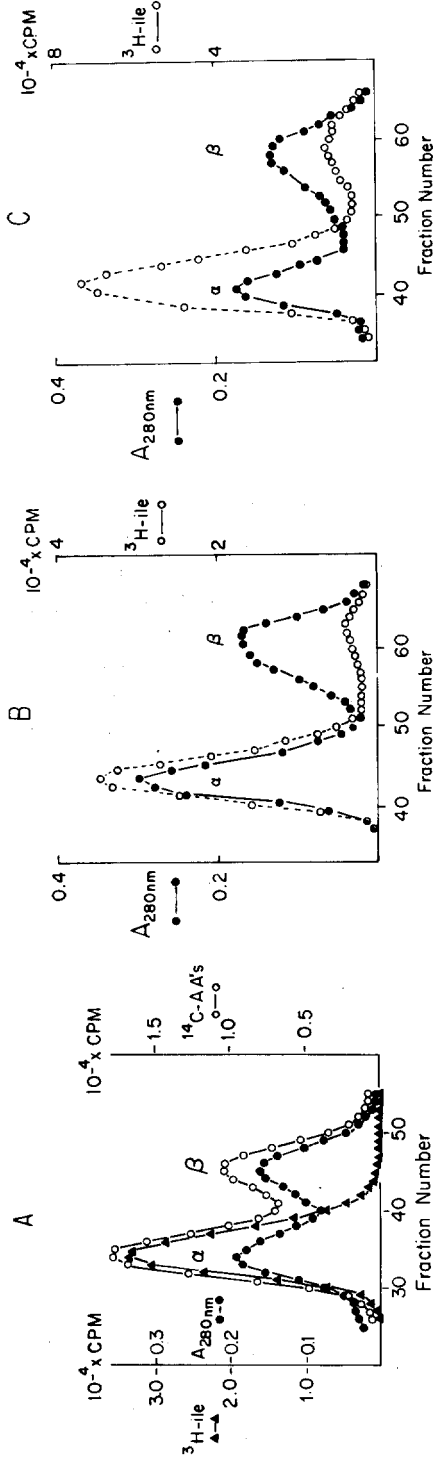† P > 0.3 by the Chi-square method for observed *vs.* expected.

FIGURE 2.—Incorporation of ³H-isoleucine into globin chains of rabbits with 0, 0.5 and 1 isoleucyl residue per chain. Rabbit reticulocytes were incubated with ³H-isoleucine, then globin from the lysates was resolved into α and β chains. The key is indicated for each part of the figure. A. Results for a rabbit with 0 isoleucyl residues in the β chain. These reticulocytes were also incubated with a ¹⁴C-amino acid mixture to show that the absence of ³H-ile counts from the β chain does not indicate a total lack of incorporation into β chains. B. Results for a rabbit with 0.5 isoleucyl residues per β chain. Incorporation for fractions 37–52 = 243,000 CPM, for 53–68 = 43,000 CPM; therefore the ratio α/β = 5.7. C. Results for a rabbit with 1 isoleucyl residue per β chain. Incorporation for fractions 35–50 = 474,000 CPM, for 51–66 = 138,000 CPM; therefore the ratio α/β = 3.4.

TABLE 3

Rabbit matings

Cross no.	Male parent Phenotype*	Genotype†	Female parent Phenotype*	Genotype†	Phenotype* 1 genotype† ile/ile number	Progeny Phenotype* 0.5 genotype† ile/val number	Phenotype* 0 genotype† val/val number
1	1	ile/ile	0	val/val	0	4	0
2	0	val/val	1	ile/ile	0	7	0
3	0	val/val	0	val/val	0	0	1
4	1	ile/ile	0.5	ile/val	2	7	0
5	0	val/val	0.5	ile/val	0	1	1
6	0.5	ile/val	0	val/val	0	1	2

* Expressed as isoleucyl residues per β chain.
† Inferred from both preceding and these results.

mixture, resulting in starvation for ile-tRNA, polyribosome patterns from the three genotypes are distinctive (Figure 3D–F). For each type there is an increase in clusters of 2 and 3 ribosomes, but the striking increase in clusters with more than 7 ribosomes for *ile/ile* is not present in *val/val*. The heterozygote *ile/val* shows an increase in the proportion of clusters of more than 7 ribosomes too, but the pattern is clearly intermediate to those for the two homozygotes. If 5 mM isoleucine is present during the incubation as well as 25 mM OMT, the polyribosome patterns are very similar to those found after incubation with the complete
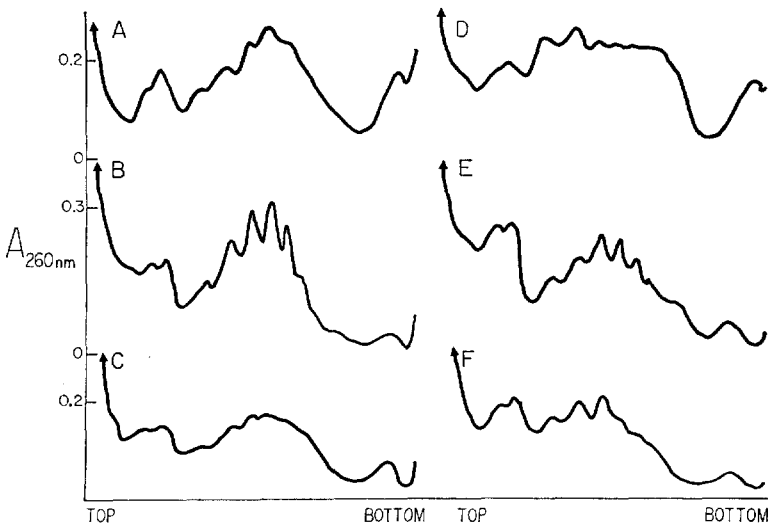

FIGURE 3.—Polyribosome patterns from rabbits with 1, 0.5 and 0 isoleucyl residues per β chain. A–C. Patterns from lysates prepared after incubating reticulocytes with a complete amino acid mixture. D–F. Patterns from lysates prepared after incubating with a mixture in which 25 mM OMT replaced isoleucine. Reticulocytes from an *ile/ile* rabbit were used for A and D; from an *ile/val* rabbit, for B and E; and from a *val/val* rabbit, for C and F.

amino acid mixture (data not shown). This reversal of the OMT effect is consistent with the observations of HORI and RABINOVITZ (1968) who showed that 5 mM isoleucine completely reverses the inhibition of amino acid incorporation induced by 25 mM OMT. The association of (1) the striking increase in the fraction of polyribosomes with more than 7 per cluster, (2) the ability of OMT to effect starvation for ile-tRNA and (3) the requirement that residue β^{112} be ile (Figure 3) fully confirms the original interpretation (HORI and RABINOVITZ 1968, FREEDMAN and RABINOVITZ 1971) that the clusters with 8–12 ribosomes form as a result of ribosomal traffic induced at the ile codon for β^{112} by the low concentration of ile-tRNA.

<div align="center">DISCUSSION</div>

The β chain polymorphism: The data on inheritance and distribution among the laboratory rabbit population permit the conclusion that the presence or absence of a residue of isoleucine at position 112 in the β chain is inherited as an autosomal codominant trait. The *1 Ile* phenotype corresponds to an *ile/ile* genotype, with *0.5 Ile* corresponding to *ile/val* and *0 Ile*, to *val/val*.

Since the *ile* allele is only slightly more frequent than *val*, this character is also polymorphic. It is extremely unlikely that a mutation has been accidently selected by a limited choice of breeding rabbits since Table 2 summarizes data on rabbits from two independent local suppliers and includes 2 Flemish Giants with *ile/ile* and *val/val* genotypes as well as 73 New Zealand Whites. Moreover, the same form of variation has been detected in rabbit colonies at many other locales (GALIZZI, WILSON, HUNTER and HONIG, individual personal communications; GARRICK and RUTLEDGE, unpublished). An ile/val substitution does not change the net charge of the β chains and is concordant with many other ile↔val interchanges in protein evolution (DAYHOFF 1972). It is difficult to argue that selective forces maintain an ile/val polymorphism in such a hydrophobic portion of the polypeptide. To suggest that this is a selectively neutral, transient polymorphism seems intuitively more appealing. Since, however, there are other residues in the β chain of rabbit hemoglobin at which variants are found (GALIZZI 1970 and 1971), one cannot rule out linkage disequilibrium combined with a polymorphism maintained by selection at one or more of these residues. The relation of the β^{112} polymorphism to these other interchanges is currently under investigation (BRICKER, HAFNER and GARRICK, unpublished). The data available do not suggest a relationship to a dimorphism described earlier (ISHIBASHI *et al.* 1968).

The ile/val polymorphism at residue β^{112} of rabbit hemoglobin adds to the list of polymorphic variants with amino acid substitutions not involving a charge change. One could systematically seek similar variants by examining the amino acid composition of a given polypeptide with special attention to whether the content of rarer amino acids varies when the polypeptide is isolated from different individuals. Favorable residues include ile in the globins, and tyr, trp, met and cys for many polypeptides. Determination of these residues is subject to technical problems like those affecting ile recovery in our work, but methods are

available for minimizing these problems (*c.f.*, KEUTMANN and POTTS 1969; LIU and CHANG 1971). In principle this approach would have detected each of the following phenomena of interest: the α^{24} tyr/phe polymorphism in horse hemoglobin (CLEGG 1970) and in deer hemoglobin (TAYLOR, EASLEY and KITCHEN 1972) and the unanticipated presence of an isoleucyl residue in Hb Lepore Hollandia (BARNABAS and MULLER 1962; CURTAIN 1964). Our approach is less laborious than determining the composition of isolated tryptic peptides; yet it should detect a sizable fraction of electrophoretically silent polymorphisms.

Reticulocyte polyribosome patterns when ile-tRNA is limiting: The association of (1) the bimodal distribution of polyribosome classes with clusters of 2 to 3 and 8 to 12 as modes, (2) starvation for ile-tRNA resulting from OMT and (3) the presence of isoleucine at β^{112} demonstrates that the larger number of ribosomes per cluster results from a traffic jam of ribosomes behind a bottleneck at the β^{112} codon for isoleucine. This interpretation has previously been supported by the enrichment in small *vs.* large clusters of ribosomes of nascent α *vs.* nascent β chains (KAZAZIAN and FREEDMAN 1968) and of α *vs.* β mRNA (TEMPLE and HOUSMAN 1972), respectively. Contrary to this interpretation was the suggestion that five ribosomes per mRNA is normally modal and seven maximal for reticulocyte lysates because the length of the Hb mRNAs in a Watson-Crick configuration will hold no more than seven ribosomal diameters (WARNER, RICH and HALL 1962). This limitation has been countered by suggesting that extensive base pairing is not permitted when ribosomes are attached to mRNA, enabling it to assume readily a more elongated configuration (FREEDMAN and RABINOVITZ 1971). Our results provide additional evidence that such an extended configuration is available to mRNA when sufficiently packed with ribosomes.

An alteration in polyribosome patterns due to rate-limiting tRNA levels has been previously predicted (BOYER, HATHAWAY and GARRICK 1964; ITANO 1967) and experimentally confirmed (HORI, RABINOVITZ and FISHER 1967; HORI and RABINOVITZ 1968). The heritability of such altered patterns has also been suggested previously and is demonstrated by our results.

Experimental utility of ile/val polymorphism: This system provides material which should permit several important experimental studies: (1) Incubating OMT with reticulocytes from *val/val* rabbits should lead to direct inhibition of α chain synthesis without a direct effect on β incorporation. Such circumstances permit evaluation of the effect of lowered α incorporation on β incorporation (GARRICK 1972; GARRICK and GARRICK 1972; HONIG, WOLF and MASON 1972). Similar studies on reticulocytes from *0.5 Ile* rabbits (RABINOVITZ *et al.* 1969) permit the inference that incorporation into the β chain with zero isoleucine is enhanced when OMT inhibits incorporation into α chains and the isoleucine-containing β chain. (2) If the erythropoietic organs of *val/val* rabbits can be starved for isoleucine without starving the entire organism, it is likely that an animal model for α-thalassemia can be produced. (3) As markers at residues 52, 56, and 76 of the β chain of rabbit hemoglobin (GALIZZI 1970) are available in our laboratory rabbits, the unexpectedly high frequency of intragenic recombination in vertebrates suggested by OHNO (1971) and perhaps already detected (GALIZZI 1971) can be verified. (4) DNA sequences corresponding to much of

the gene for the (α and) β chain(s) of rabbit hemoglobin have been prepared (KACIAN *et al.* 1972; Ross *et al.* 1972; VERMA *et al.* 1972). With such material from *ile/ile* rabbits, *val/val* rabbits or their erythropoietic cells would serve as an excellent assay system for the feasibility of gene substitution (genetic transformation).

LITERATURE CITED

BARNABAS, J. and C. J. MULLER, 1962 Haemoglobin-Lepore Hollandia. Nature **194**: 931–932.

BEST, J. S., V. FLAMM and G. BRAUNITZER, 1969 The primary structure of the β-chain of rabbit haemoglobin. Z. Physiol. Chem. **350**: 563–580.

BORSOOK, H., C. L. DEASY, A. J. HAAGEN-SMIT, G. KEIGHLEY and P. H. LOWY, 1952 Incorporation in vitro of labeled amino acids into proteins of rabbit reticulocytes. J. Biol. Chem. **196**: 660–694.

BORSOOK, H., E. H. FISCHER and G. KEIGHLEY, 1957 Factors affecting protein synthesis in vitro in rabbit reticulocytes. J. Biol. Chem. **229**: 1059–1070.

BOYER, S. H., 1972 Extraordinary incidence of electrophoretically silent genetic polymorphisms. Nature **239**: 453–454.

BOYER, S. H., D. C. FAINER and M. A. NAUGHTON, 1963 Myoglobin: Inherited structural variation in man. Science **140**: 1228–1231.

BOYER, S. H., P. HATHAWAY and M. D. GARRICK, 1964 Modulation of protein synthesis in man: An *in vitro* study of hemoglobin synthesis by heterozygotes. Cold Spring Harbor Symp. Quant. Biol. **34**: 333–346.

BRAUNITZER, G., J. S. BEST, U. FLAMM and B. SCHRANK, 1966 Zur Phylogenie des Hämoglobins: Untersuchungen am Hämoglobin des Kaninchens (*Caniculus*). Z. Physiol. Chem. **347**: 207–211.

BRAUNITZER, G., V. FLAMM, J. S. BEST and B. SCHRANK, 1968 Zur Phylogenie des Hämoglobinmoleküls. Die Konstitution der α-Ketten des Hämoglobins des Kaninchens (*Caniculus*). Z. Physiol. Chem. **349**: 1073–1075.

BRICKER, J. and M. D. GARRICK, 1973 An isoleucine-valine substitution in the β chain of rabbit hemoglobin. (In preparation.)

CLEGG, J. B., 1970 Horse haemoglobin polymorphism: evidence for two linked non-allelic α-chain genes. Proc. Roy. Soc. Lond. B **176**: 235–246.

CURTAIN, C. C., 1964 A structural study of abnormal haemoglobins occurring in New Guinea. Australian J. Exptl. Biol. **42**: 89–97.

DAYHOFF, M. O., 1972 *Atlas of Protein Sequence and Structure 1972 Vol. 5* National Biomedical Research Foundation, Washington.

DINTZIS, H. M., 1961 Assembly of the peptide chains of hemoglobin. Proc. Natl. Acad. Sci. U.S. **47**: 247–261.

FREEDMAN, M. L. and M. RABINOVITZ, 1971 Structure of the large β-chain polyribosome of rabbit reticulocytes rendered isoleucine-deficient by O-methylthreonine. Molec. Pharmacology **7**: 317–322.

GALIZZI, A., 1970 The amino acid sequence of the β-chains of rabbit hemoglobin. Eur. J. Biochem. **17**: 49–55. ——, 1971 Polymorphic alleles of the gene for the β-chain of rabbit haemoglobin. Nature New Biol. **229**: 142–143.

GARRICK, L. M., 1972 Transfer RNA dependent control of hemoglobin synthesis. Ph.D. dissertation, Department of Biology, University of Virginia, Charlottesville, Virginia.

GARRICK, L. M. and M. D. GARRICK, 1972 Increased β globin chain synthesis resulting from decreased α synthesis. Am. Soc. Hematol. **15**: 92.

GARRICK, M. D., P. DEMBURE and R. GUTHRIE, 1973 Sickle-cell anemia and other hemoglobinopathies. Procedures and strategy for screening employing spots of blood on filter paper as specimens. New Eng. J. Med. **288**: 1265–1268.

HARRIS, H., 1966 Enzyme polymorphisms in man. Proc. Roy. Soc. Lond. B. **164**: 298–310.

———, 1971 *The principles of human biochemical genetics.* pp. 12–16. American Elsevier, New York.

HONIG, G. R., J. L. WOLF and R. G. MASON, 1972 Dependence of hemoglobin β chain synthesis on synthesis of α chains by bone marrow cells. Amer. Soc. Hematol. **15**: 93.

HORI, M., J. M. FISHER and M. RABINOVITZ, 1967 Polyribosomes during interrupted growth of hemoglobin chains. Science **155**: 83–84.

HORI, M. and M. RABINOVITZ, 1968 Polyribosomal changes during inhibition of rabbit hemoglobin synthesis by an isoleucine antagonist. Proc. Natl. Acad. Sci. U.S. **59**: 1349–1355.

HUBBY, J. L. and R. C. LEWONTIN, 1966 A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura.* Genetics **54**: 577–594.

ISHIBASHI, S., L. C. NIU, Y. H. YUH, M. C. NIU and E. B. KALAN, 1968 Evidence for dimorphism in rabbit hemoglobins. Proc. Soc. Exptl. Biol. Med. **128**: 879–885.

ITANO, H. A., 1967 Genetic regulation of peptide synthesis in hemoglobins. J. Cell Physiol. **67** (Suppl. 1) 65–76.

KACIAN, D. L., S. SPIEGELMAN, A. BANK, M. TERADA, S. METAFORA, L. DOW and P. MARKS, 1972 *In vitro* synthesis of DNA components of human genes for globins. Nature New Biol. **235**: 167–169.

KAZAZIAN, H. and M. L. FREEDMAN, 1968 The characterization of separated α and β chain polyribosomes in rabbit reticulocytes. J. Biol. Chem. **243**: 6446–6450.

KEUTMANN, H. T. and J. T. POTTS, JR., 1969 Improved recovery of methionine after acid hydrolysis using mercaptoethanol. Analyt. Biochem. **29**: 175–185.

LEWONTIN, R. C. and J. L. HUBBY, 1966 A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura.* Genetics **54**: 595–609.

LIU, T. Y. and Y. H. CHANG, 1971 Hydrolysis of proteins with p-toluenesulfonic acid. Determination of tryptophan. J. Biol. Chem. **246**: 2842–2848.

OHNO, S., 1970 *Evolution by Gene Duplication.* pp. 50–53. Springer-Verlag, New York.

ORNSTEIN, L. and B. J. DAVIS, 1964 Disc electrophoresis—I. Background and theory.—II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**: 321–349, 404–427.

RABINOVITZ, M., M. L. FREEDMAN, J. M. FISHER and C. R. MAXWELL, 1969 Translational control of hemoglobin synthesis. Cold Spring Harbor Symp. Quant. Biol. **34**: 567–578.

RABINOVITZ, M. and J. M. FISHER, 1964 Characteristics of the inhibition of hemoglobin synthesis in rabbit reticulocytes by threo-α-amino-β-chlorobutyric acid. Biochim. Biophys. Acta **91**: 313–322.

ROSS, J., H. AVIV, E. SCOLNICK and P. LEDER, 1972 *In vitro* synthesis of DNA complementary to purified rabbit globin mRNA. Proc. Natl. Acad. Sci. U.S. **69**: 264–268.

SMULSON, M. E. and M. RABINOVITZ, 1968 L-O-methylthreonine—Synthesis and mode of action as an isoleucine antagonist. Arch. Biochem. Biophys. **124**: 306–313.

TAYLOR, W. J., C. W. EASLEY and H. KITCHEN, 1972 Structural evidence for heterogeneity of two hemoglobin α chain gene loci in white-tailed deer. J. Biol. Chem. **247**: 7320–7324.

TEMPLE, G. F. and D. E. HOUSMAN, 1972 Separation and translation of the mRNAs coding for α and β chains of rabbit globin. Proc. Natl. Acad. Sci. U.S. **69**: 1574–1577.

VERMA, I. M., G. F. TEMPLE, H. FAN and D. BALTIMORE, 1972 *In vitro* synthesis of DNA complementary to rabbit reticulocyte 10s RNA. Nature New Biol. **235**: 163–167.

VON EHRENSTEIN, G., 1966 Translational variations in the amino acid sequence of the α-chain of rabbit hemoglobin. Cold Spring Harbor Symp. Quant. Biol. **31**: 705–714.

WARNER, J. R. and A. RICH, 1964 The number of soluble RNA molecules on reticulocyte polyribosomes. Proc. Natl. Acad. Sci. U.S. **51**: 1134–1141.

WARNER, J. R., A. RICH and C. E. HALL, 1962 Electron microscope studies of ribosomal clusters synthesizing hemoglobin. Science **138**: 1399–1403.