the muscles, and still there were no effects noted. A solution containing 3 mg of curare per milliliter was injected into the abdomen of the fly by means of a 31-gauge hypodermic needle coupled to a microliter syringe. Injection could be carried out without dislodging the recording microelectrode. This technique allowed continuous observation and recording of the curare effect. The complete sequence of events—that is, the control, the treatment with curare, and the recovery—could thus be recorded in a single fiber with the microelectrode undisturbed.

The injection of 0.01 ml of *d*-tubocurarine chloride solution (30 μg) into the abdomen of the fly resulted in a typical vertebrate-type neuromuscular block. The spike-like portion of the action potential gradually diminished in amplitude until only a small, slow, graded-type potential remained. The sequential effects of curare as a neuromuscular blocking agent are illustrated in Fig. 1. Figure 1A shows the normal intracellular response, and B–G show the progressive decrement effected by curare. The resting potential was not affected; it held steady and stable throughout the entire experimental period.

The effectiveness of curare as a neuromuscular blocking agent in insects is apparently related to the route of administration, and this factor may be a reflection of the penetrability of the substance into the target area. The basement membrane, a laminar structure similar to cuticle, invests many insect tissues and is proposed to serve as a diffusion barrier to ions and pharmocologic agents (*3*). The interposition of this tissue structure around the end-plate region as a restrictive factor to the passage of curare molecules appears to be circumvented by the intra-abdominal administration.

However, other possible explanations must be considered. Curare may combine with a substance from the tissues of the abdomen to form a complex intermediate, or it may combine with a component of the circulatory system to produce a substance specifically toxic at the end-plate region.

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Hemoglobin Freiburg: Abnormal Hemoglobin Due to Deletion of a Single Amino Acid Residue

Abstract. *Structural characterization of a new variant of human hemoglobin (adult), designated hemoglobin Freiburg, indicates the deletion of the valyl residue No. 23 from an otherwise normal beta-chain. The formula may be written* $\alpha_2\beta_2^{23\text{val}-0}$. *The abnormal hemoglobin is present with hemoglobin A in the proposita and in two of her three living children, but is not detectable in her parents. We postulate that this variant represents a triplet base deletion which most likely resulted from an unequal crossing-over between two normal beta-chain loci during meiosis in one of the parents of the proposita.*

Chemical and genetic studies of human hemoglobins (Hb) have contributed substantially to modern concepts of molecular biology. The discovery of Hb S by Pauling, Itano, Singer, and Wells (*1*) was the first direct evidence that an abnormal gene could alter the physical-chemical properties of a protein. Ingram's structural characterization of Hb S demonstrated that the genetic change responsible for this abnormal hemoglobin resulted in the substitution of one amino acid residue by another residue at a single site of the protein molecule (*2*). About 50 or more analogous examples of single amino acid substitutions have been observed among other abnormal human hemoglobins (*3, 4*). Evidence has recently been presented that two amino acid substitutions may be present in the β-chain of one abnormal hemoglobin, Hb Cf-Georgetown (*5*). This abnormality may have resulted from two single-point mutations affecting the same gene or from an intragenic equal crossing-over between two different homologous, mutant genes. In addition, evidence of intergenic unequal crossing-over has been obtained from chemical study of Hb Lepore (*6, 7*). We now describe chemical and genetic studies of Hb Freiburg, which is the first example of an abnormal protein in which the deletion of a single amino acid has been recognized. These studies support the theory that intragenic unequal crossing-over accounts for this mutation.

Hemoglobin Freiburg was discovered by Betke and co-workers (*8*) in a German woman in whom they found an associated hemolytic process and mild cyanosis. Figure 1 represents the family pedigree in which the proposita is represented as II-2. Both Hb Freiburg and slight cyanosis were also found in two of her three living children, III-3 and III-4, but the children showed no evidence of hemolysis. The abnormal hemoglobin represents from 27 to 32 percent of the total hemoglobin in the affected individuals. Except for 2.5 to 3.0 percent of Hb A_2, the remaining heme protein appears to be normal adult hemoglobin. A comparison of Hb Freiburg with other hemoglobins by starch-gel electrophoresis (*3*) is shown in Fig. 2. Its electrophoretic mobility is less than that of Hb A, greater than that of Hb S, and very similar to that of Hb F (normal fetal hemoglobin) at *p*H 8.1.

Hemoglobin Freiburg was not detected in the parents of the proposita, I-1 and I-2, nor in her three brothers, II-3, II-4, and II-5, nor in her oldest child, III-1. Her second child, III-2, died as a newborn from a hemolytic disease. Hemoglobin studies were not performed at that time. Analysis of blood groups does not exclude I-1 and I-2 as parents of the proposita, II-2.

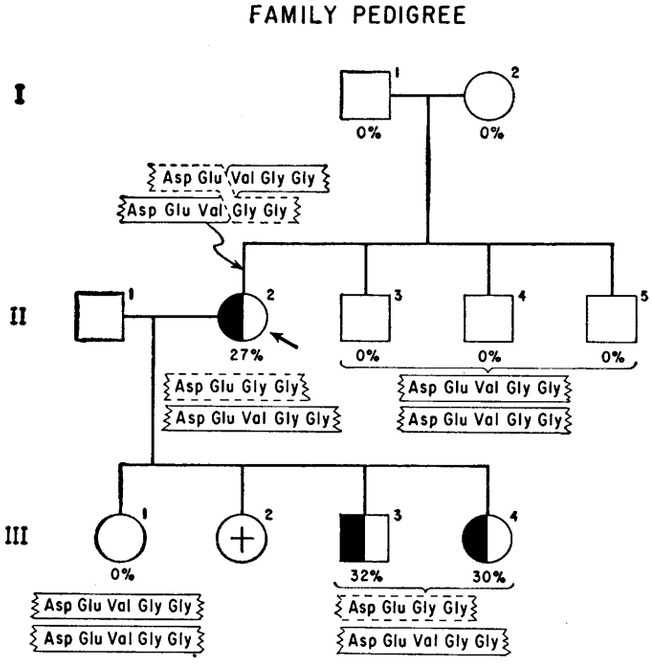
The oxygen dissociation curve of blood containing Hb Freiburg is displaced to the left, an indication of greater than normal affinity for oxygen (*9*). This shift was thought to be due, at least in part, to the presence of about 10 percent of the total hemeprotein as methemoglobin. The spectral

curve of the hemoglobin in the hemolyzate of erythrocytes from the proposita is presented in Fig. 3. The spectra of purified ferrihemoglobin Freiburg appear to be unique, differing from Hb A and the known Hb M variants (*10*). Studies of subunit hybridization of Hb Freiburg with Hb S, which is abnormal in the β-chain, failed to produce hybrid hemoglobins. Similar studies of Hb Freiburg with a Hb D, abnormal in the α-chain, resulted in the formation of normal Hb A. These studies indicate that the α-chain of Hb Freiburg is normal and thus that the β-chain is abnormal (*3*). The rate of heat denaturation of Hb Freiburg in the ferrihemoglobin cyanide form was more than twice as rapid as that of Hb A.

Table 1. Amino acid compositions of tryptic peptides of the β-chain of hemoglobin Freiburg.

Tryptic peptide	Zone No.	Composition in ratios of amino acid residues
βT-1	X	($Thr_{0.9}$, $Glu_{2.0}$, $Pro_{1.1}$, $Val_{1.0}$, $Leu_{1.0}$, $His_{1.0}$, $Lys_{1.0}$)
βT-2	VIII	($Thr_{1.0}$, $Ser_{0.9}$, $Gly_{1.1}$, $Ala_{2.1}$, $Val_{1.0}$, $Leu_{1.1}$, $Try_{1.0}$, $Lys_{1.0}$)
βT-3 (HCl)	IV	($Asp_{1.0}$, $Glu_{2.0}$, $Gly_{3.0}$, $Ala_{1.1}$, $Val_{2.0}$, $Leu_{1.0}$, $Arg_{1.0}$)
βT-3 (LAP)	IV	($Asp_{1.2}$, $Asn_{0.9}$, $Glu_{2.0}$, $Gly_{2.0}$, $Ala_{1.1}$, $Val_{2.0}$, $Leu_{1.1}$, $Arg_{0.8}$)
βT-4	VII	($Thr_{1.0}$, $Glu_{1.0}$, $Pro_{1.2}$, $Val_{2.0}$, $Leu_{2.1}$, $Tyr_{0.8}$, $Try_{1.0}$, $Arg_{1.0}$)
βT-5	I	($Asp_{3.0}$, $Thr_{1.0}$, $Ser_{2.0}$, $Glu_{1.1}$, $Pro_{2.0}$, $Gly_{2.2}$, $Ala_{1.1}$, $Val_{1.1}$, $Met_{0.8}$, $Leu_{1.0}$, $Phe_{2.7}$, $Lys_{1.0}$)
βT-6	XIV	($Val_{1.0}$, $Lys_{1.1}$)
βT-7	XVIII	($Gly_{1.1}$, $Ala_{0.9}$, $His_{1.0}$, $Lys_{1.0}$)
βT-8	X	(Lys)
βT-9	VI	($Asp_{3.0}$, $Ser_{0.9}$, $Gly_{2.0}$, $Ala_{2.0}$, $Val_{1.0}$, $Leu_{4.0}$, $Phe_{1.0}$, $His_{1.0}$, $Lys_{1.0}$)
βT-10	XII	($Asp_{1.0}$, $Thr_{1.0}$, $Ser_{1.0}$, $Glu_{1.0}$, $Gly_{1.1}$, $Ala_{1.0}$, $Leu_{1.9}$, $Phe_{1.0}$, $AECys_{0.8}$, $His_{0.9}$, $Lys_{1.0}$)
βT-11	XI	($Asp_{2.0}$, $Glu_{1.0}$, $Pro_{1.0}$, $Val_{1.0}$, $Leu_{1.0}$, $Phe_{1.0}$, $His_{1.0}$, $Arg_{1.0}$)
βT-12a	V	($Asp_{1.1}$, $Gly_{1.1}$, $Val_{1.9}$, $Leu_{3.0}$, $AECys_{0.7}$)
βT-12b	XIX	($Gly_{1.0}$, $Ala_{1.0}$, $Val_{0.9}$, $Leu_{1.0}$, $Phe_{1.0}$, $His_{2.0}$, $Lys_{1.0}$)
βT-13	II	($Thr_{1.0}$, $Glu_{3.0}$, $Pro_{1.9}$, $Ala_{2.1}$, $Val_{1.0}$, $Tyr_{1.0}$, $Phe_{1.0}$, $Lys_{1.0}$)
βT-14	VII	($Asp_{1.0}$, $Gly_{1.1}$, $Ala_{3.9}$, $Val_{2.7}$, $Leu_{1.0}$, $His_{1.0}$, $Lys_{1.0}$)
βT-15	XV	($Tyr_{1.0}$, $His_{1.1}$)

Structural characterization of Hb Freiburg was carried out by first isolating the abnormal component by column chromatography on carboxymethyl cellulose or diethylaminoethyl-Sephadex (*11*). The hemoglobin was converted to globin by treatment with cold acidified acetone. The α- and β-chains were separated by chromatography on carboxymethyl cellulose in the presence of 8*M* urea (*12*). The β-chain was aminoethylated with ethylenimine (*13*) and digested with trypsin for 2 hours in the presence of trimethylamine buffer (*7*). The tryptic peptides were separated by automatic column chromatography with the short column (0.9 by 17 cm, Spinco 15A resin) of an automatic amino acid analyzer (Spinco Model 120) and a linear gradient of pyridine acetic acid developer (*13*). The peptide chromatogram is illustrated in Fig. 4 and is identical to that of normal β-chain tryptic peptide except for the fourth zone (IV) (*13*, *14*). Material in all zones was further purified by rechromatography on columns of Ami-



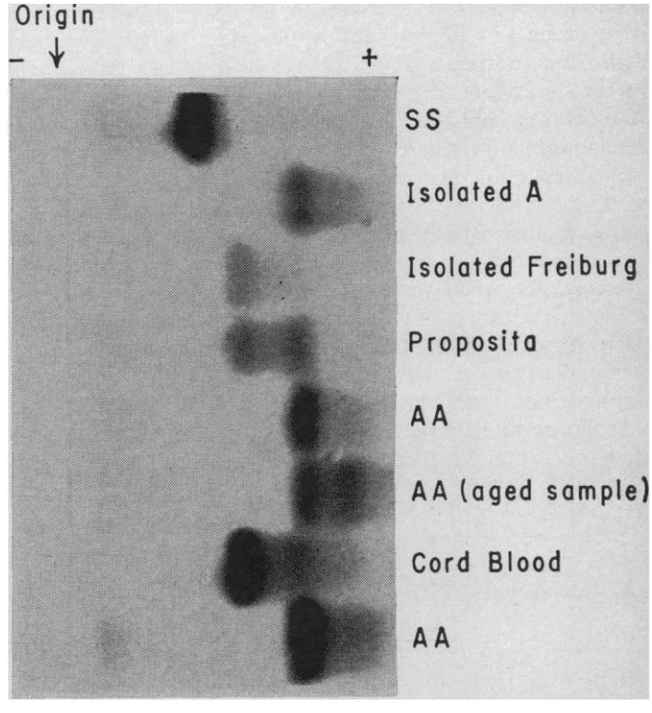


Fig. 1 (left). The proposita, II-2, is indicated by an arrow. Those individuals in whom Hb Freiburg has been detected are represented by half-shaded symbols. The percentage of Hb Freiburg which was determined by carboxymethyl-cellulose chromatography (*11*) is indicated below the symbol for each individual. Child III-2 died at birth from hemolytic disease before our hemoglobin studies were made. A meiotic unequal crossing-over, occurring in either I-1 or I-2 and giving rise to the partially deleted gene for Hb Freiburg, is indicated symbolically above II-2 by use of the three-letter abbreviation of amino acids to designate the DNA codons for residue Nos. 21 through 25 of the β-chain gene. The genotypes of several family members are indicated by use of the same code designation. Fig. 2 (right). Starch-gel electrophoresis of the hemoglobin of the proposita and of chromatographically isolated Hb Freiburg compared with hemoglobins A, S, and F.

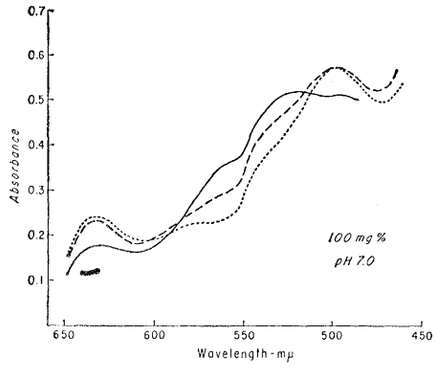


Fig. 3. Visible spectra of ferrihemoglobin forms of Hb A hemolyzate (dotted line), Hb A and Freiburg hemolyzate (dashed line), and isolated Hb Freiburg (solid line).

nex Ag 50W-X2 (Bio-Rad Laboratories) in order to obtain single, pure peptides. Quantitative amino acid analyses were made for each tryptic peptide after hydrolysis for 22 to 70 hours at 110°C in 6*N* HCl (Table 1) (*15*). The composition of each peptide is given in terms of ratios of amino acid residues to the nearest tenth. Contaminants of less than 0.14 are not included in the table. Only the composition of peptide βT-3 from Hb Freiburg (zone IV) is abnormal compared with the normal βT-3 (*16*). Repeated analyses after hydrolysis with HCl as well as after hydrolysis with leucine aminopeptidase (*17*) revealed the absence of one of three valines without the presence of an extra amino acid residue. Specifically, tryptophan and derivatives of cysteine such as *S*-aminoethyl cysteine were not present.

Determination of the amino-terminal sequence of the βT-3 peptide by a modification of the Edman phenylthiohydantoin procedure (*18*) was successful for three residues and revealed that the normal sequence Val-Asn-Val was present (*15*). A peptide with the composition (Val, Asn, Val) was also obtained from the abnormal βT-3 peptide after partial hydrolysis in refluxing 0.25*M* acetic acid for 9 hours. The amino acid composition of the remaining portion of βT-3 and the compositions of the other tryptic peptides are consistent with a normal β-chain. It is significant that pepsin, which cleaves normal βT-3 at valine No. 23, does not hydrolyze Freiburg βT-3 at this position under the same conditions, but gives a larger yield of (Leu, Gly, Arg) peptide.

We conclude from the amino acid compositions and limited studies of the amino acid sequence of the abnormal βT-3 peptide that the valyl residue normally present as residue No. 23 of the β-chain, is absent. Because there is no evidence of a substitution of this valyl residue by any other ordinary amino acid residue we conclude that the normal valyl residue 23 has been deleted from the β-chain of Hb Freiburg. The data also demonstrate that sequences unique to the δ-chain of Hb A_2 are not present on either the NH_2-terminal or COOH-terminal side of the deleted region.

The absence of the valyl residue No. 23 from the β-chain of Hb Freiburg appears most likely to be due to the deletion of the corresponding nucleotide base triplet from the gene which determines the structure of the normal β-chain. The genetic event leading to the deletion of a base triplet in Hb Freiburg may have been analogous to the mechanism which results in single and double base deletions and additions in acridine mutants of microorganisms (*19*). It has been proposed that these mutants arise as the result of unequal crossing-over between homologous genes (*20*). The presence of two glycyl residues immediately adjacent to the deleted valyl region in the β-chain may represent the site at which nonhomologous pairing occurred prior to unequal crossing-over (*21*). Because the proposita was the first member of her family to manifest Hb Freiburg, the intragenic unequal crossing-over must have occurred in the germ cells of one of her parents during meiosis.

Two other explanations for the apparent deletion of valyl residue No. 23 have been considered and include (i) a substitution of the valyl residue by some unusual and, as yet, undetected α-amino acid residue and (ii) a single codon change which fails to insert any residue at position No. 23 but does result in the formation of a peptide bond between positions 22 and 24. Both of these explanations have been rejected on the basis of current concepts of the genetic control of protein synthesis.

The deletion of an amino acid in Hb Freiburg is of both evolutionary and chemical interest. The mechanism by which Hb Freiburg arose may be the same as that which is responsible for the formation of "sequence gaps" observed earlier by other investigators who have compared the amino acid sequences of the α- and β-chains of hemoglobin and myoglobin of various species (*22*). From a chemical point of view, it is interesting to note that the deletion of a neutral residue from the B-helix of the β-chain results in a change in electrophoretic mobility and slight-to-moderate changes in other properties of the hemoglobin. Although the B-helix is not next to the heme group of the β-chain, the alteration in structure due to the deletion appears to modify the influence of the globin on its heme, as judged by the change in absorption spectra and stability of Hb Freiburg in the oxidized form. Further studies of the chemical proper-

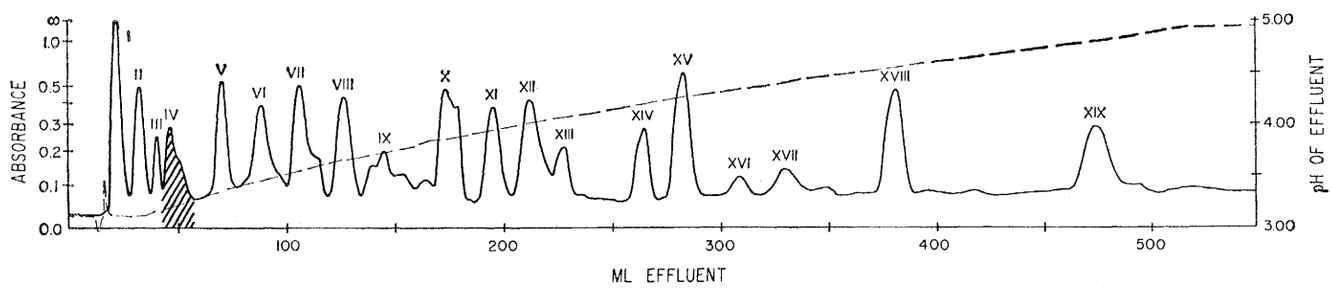


Fig. 4. Peptide pattern of tryptic hydrolyzate of aminoethylated β-chains from Hb Freiburg. The absorbance (solid line) of the reaction products of ninhydrin and peptides was measured continuously at 570 mμ with an automatic amino acid analyzer. The *p*H of the effluent was measured continuously and is represented by the dashed line. Zones are designated by roman numerals. Except for the extra zone IV, which is shaded, this peptide pattern is identical to that of normal β-chain.

ties of this abnormal hemoglobin, in view of its unusual structure, may be helpful in understanding more about the relation of chemical structure to chemical function of hemoglobin.

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14. The peptide zones in Fig. 3 are designated by roman numerals in order of their elution. The nomenclature of tryptic peptides (Table 1) includes designation of hemoglobin chain (in this case β-chain), the type of peptide (in this case T for tryptic peptide), and position of the peptide from the amino-terminal end of the polypeptide chain. Designation of the position is by arabic numerals.
15. Amino acid residues are abbreviated as follows: Ala, alanine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Cys, cysteine; Glu, glutamic acid; His, histidine; Gly, glycine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Try, tryptophan; Tyr, tyrosine; Val, valine; AE, aminoethyl.
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23. We thank the family in which this hemoglobin variant occurs for cooperation. We thank R. D. Koler with whom we first discussed the possible genetic mechanisms which might have given rise to Hb Freiburg. The technical help of M. Duerst, R Wright, and A. Dozy is appreciated. Supported by USPHS grants CA-07941 and H-5168 and by grant of Deutsche Forschungsgemeinschaft.

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Co-linearity of β-Galactosidase with Its Gene by Immunological Detection of Incomplete Polypeptide Chains

Abstract. *Many nonsense mutants that map in the β-galactosidase structural gene produce material that forms precipitin lines when tested by double diffusion on agar against antiserum prepared from native β-galactosidase. Relative sizes of the cross-reacting materials measured by sucrose density gradient centrifugation are the same as sizes calculated from genetic mapping of nonsense mutants. Orientation of the protein to its gene is also indicated.*

Co-linearity of gene and protein has been elegantly demonstrated by Yanofsky and his co-workers for the tryptophan synthetase A protein of *Escherichia coli* (*1*) and by Sarabhai, Stretton, Brenner, and Bolle, who used the coat protein of phage T-4A (*2*). In the latter case, positions of mutations in a series of nonsense mutants in the structural gene for coat protein of the phage were ordered by genetic methods. The prematurely terminated polypeptide chain produced by each mutant strain was shown to be proportional in length to the position of mutation.

These ingenious experiments were feasible because the coat protein comprises more than 90 percent of all protein produced on infection. We describe experiments with β-galactosidase that are similar in concept to those of Sarabhai *et al.*, but in our study incomplete polypeptide chains comprised at most a few percent of the protein in nonsense mutant strains. We found that many of these cross-react with antiserum to β-galactosidase. This immunological test allowed detection of incomplete chains after sucrose density gradient centrifugation and comparison of the relative sizes of these chains with the expected size, based on the position of mutation within the gene.

Genetic mapping of the nonsense mutants used in our study was reported by Newton, Beckwith, Zipser, and Brenner (*3*). These strains and a wild-type *E. coli* (Hfr 3000) were grown with aeration at 37°C to the exponential phase on 0.4 percent glycerol in medium 63 (*4*) containing 1 μg of thiamine and, for strains of the NG series, 20 μg of tryptophan per milliliter. Cultures were induced for the lactose operon by addition of $1 \times 10^{-3}M$ β-D-isopropyl thiogalactoside (IPTG) to mutants in areas 1 to 10 (Table 1) or $5 \times 10^{-4}M$ IPTG for mutants in areas 11 to 16 and for the wild type. After approximately four doublings (each 90 to 120 minutes), cells were harvested, suspended in a density of 2 to 4×10^{11} cells per milliliter in 0.05*M* potassium phosphate buffer (*p*H 7.2), and broken in the cold with a Branson sonifier. Clear supernatants were obtained by centrifugation at 48,000*g* for 30 minutes.

The precipitin reaction was carried

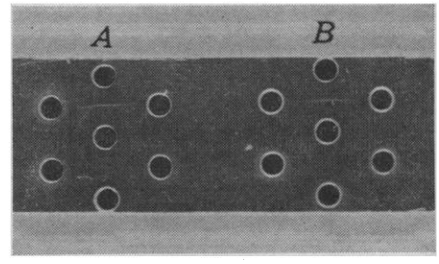

Fig. 1. Precipitin lines produced with antiserum to β-galactosidase. Ten microliters of antiserum were placed in each center well, and β-galactosidase (1.5 μg) was added to wells *A* and *B*. Proceeding clockwise from *A*, 10 μl of extracts of NG 125, U 366, X 90, YA 559, and NG 200, and clockwise from *B*, extracts of X 90, U 366, uninduced X 90, NG 200, and NG 125 were added.