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.

30 September 1966

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 $0.05M$ 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

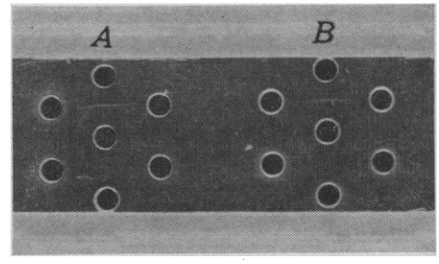

Fig. 1. Precipitin lines produced with antiserum to β-galactosidase. Ten microliters of antiserum were placed in each center well, and β-galactosidase (1.5 μg) was added to wells *A* and *B*. Proceeding clockwise from *A*, 10 μl of extracts of NG 125, U 366, X 90, YA 559, and NG 200, and clockwise from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

Table 1. Specific activities of cross-reacting material (CRM), sedimentation constants, and approximate molecular weights of incomplete polypeptide chains produced by β-galactosidase nonsense mutants. Area 1 is adjacent to the operator site and area 16 is next to the permease gene in the lactose operon.

Mutant	Type	Area	CRM (unit/mg of protein)	Thiogalactoside transacetylase (unit/mg of protein)	$s_{20,w}$	Mol. wt. (calc. from $s_{20,w}$)	Mol. wt. (calc. from genetic mapping)
U 118	Ochre	1	0	0.01			
YA 486	Amber	3	8	.04			
NG 125	Amber	6	38	.1	4.2	60,000	67,000
YA 559	Amber	8	10	.1			
NG 750	Amber	10	4	.3			
NG 200	Amber	11	31	.3	5.5	91,000	101,000
U 366	Amber	13	38	1.0	6.0	103,000	111,000
X 90	Ochre	16	52	2.6	6.6	122,000	127,000

out on agar-coated microscope slides. Ten microliters of antiserum to β-galactosidase (*5*) were placed in the center well and the same volume of the supernatant to be tested was added to an outer well. Presence of precipitin lines (see Fig. 1) was determined by visual examination after 16 hours at 25°C. The β-galactosidase complex produces a sharp line, while lines produced with extracts of nonsense mutants are more diffuse and these fuse with spurring to the β-galactosidase line to indicate antigenic similarity. Further evidence for antigenic similarity was obtained by treatment of the antiserum with β-galactosidase. After removal of the precipitate, composed of β-galactosidase and antiserum to β-galactosidase, by centrifugation, no precipitin lines were formed when the supernatant was tested against several mutant extracts. Quantitation was achieved by serial dilution of the extract in phosphate buffer. One unit of cross-reacting material (CRM) was defined as the minimum amount in 0.01 ml that produced a visible precipitin line. Specific activity is in units per milligram of protein. We estimate that the precision of this measurement is ± 25 percent, but specific activity of CRM in extracts obtained from a single mutant occasionally varied from culture to culture by as much as 100 percent.

Mean values of CRM activity produced by eight different nonsense mutants are shown in Table 1. We used the designation of Newton *et al.* (*3*) in which the β-galactosidase structural gene is divided into 16 areas, from 1, near the operator region, to 16, just before the permease structural gene. Mutants NG 200, U 366, and X 90, mapped in areas 11, 13, and 16, respectively, form relatively high levels of CRM; no detectable CRM was formed by strain U 118 (area 1), and low levels were produced by strains YA 486, YA 559, and NG 750 (areas 3, 8, and 10). Mutant NG 125 (area 6) produced a level of CRM as high as the level produced by strains in which the position of mutation is toward the permease end of the gene. Table 1 also shows analyses for thiogalactoside transacetylase (*6*); these were in full agreement with values reported earlier (*3*). Extracts were checked routinely for absence of β-galactosidase activity. No precipitin lines were found when extracts from noninduced cultures of mutants or of wild type were tested, except for strain U 366 (area 13) which is constitutive for the lactose operon. For purposes of comparison, the induced wild-type strain Hfr 3000 was tested and found to have a specific activity of 240. Under the same conditions of assay, 0.15 μg of pure β-galactosidase (*7*) gave a visible precipitin line.

To determine approximate sizes of the polypeptide chains, crude extracts (0.2 ml) from four strains with the highest specific activity were layered above 5 to 20 percent sucrose gradients (*8*) containing, in 5 ml, 0.01*M* tris, 0.2*M* NaCl, and 0.005*M* mercaptoethanol, *p*H 7.0. Human hemoglobin (1 mg) was added as a reference marker, and the gradients were centrifuged at 38,000*g* for 5 to 16 hours at 3°C. Tubes were punctured at the bottom, and fractions of 2 drops each were collected and tested quantitatively for CRM. Sedimentation constants were obtained by comparison with hemoglobin, assuming 4.5*S* for the latter. Mean sedimentation constants for NG 125, NG 200, U 366, and X 90 (areas 6, 11, 13, and 16) obtained from three or more determinations for each mutant are shown in Table 1. Approximate molecular weights were calculated by the method of Martin and Ames (*8*) from $s_{20,w}$ values for the mutant and for hemoglobin (molecular weight 66,000).

β-Galactosidase is a tetramer of 16*S* with a molecular weight of 540,000 (*9*). Since the minimum molecular weight is 135,000 (*10*), the structural gene must be of a length sufficient to code for a monomer of this size. We calculated the expected molecular weight of each of the incomplete polypeptide chains from the genetic data given by Newton *et al.* (*3*). The position of mutation in strain NG 125 (area 6) is in the center of the gene, and a polypeptide chain of 67,000 should be produced; a value of 60,000 was obtained from sedimentation data. Similarly, the corresponding molecular weights for strain X 90 (area 16) are 127,000 and 122,000. Data for the two other mutants tested also are in agreement. Therefore, these experiments demonstrate co-linearity of gene and protein. It is also evident that incomplete polypeptide chains exist in the extracts in nonaggregated form.

Since synthesis of a polypeptide chain occurs in a direction proceeding from amino- to carboxyl-terminal (*11*), these data provide evidence not only for co-linearity, but also for orientation of the protein to its gene in such a manner that the coding sites for amino- and carboxyl-terminal ends of β-galactosidase correspond to operator and permease ends of the structural gene, respectively. Absence of CRM in extracts of strain U 118 (area 1), which is expected to produce a relatively short polypeptide chain lacking antigenic determinants, is also in accord. The same conclusion, with regard to orientation, has been drawn from experiments which showed that the amino-terminal dipeptide of β-galactosidase could be isolated from crude extracts of induced mutant X 90 (*12*).

It seems unlikely that variations in extent of the antigen-antibody reaction from mutant to mutant reflect differences in rates of synthesis of each incomplete polypeptide chain. We believe it more likely that rates of synthesis of each chain are the same and that antigenic reactivity is influenced primarily by the sequence of amino acids produced, which allows a folding into a three-dimensional configuration

either more or less like the native protein. According to this interpretation, incomplete polypeptide chains produced by mutants YA 559 and NG 750 (areas 8 and 10), although presumably longer than that formed by NG 125 (area 6), would be folded less like the native. This implies further that these incomplete chains exist not in random configuration but in specific conformation. In addition, it is possible that the antibody itself may serve as a directing influence in folding (*13*).

Production of CRM by nonsense mutants is generally not observed in other systems. Since β-galactosidase is a very large protein, however, it is not surprising that many incomplete polypeptide chains retain antigenic determinants.

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14 October 1966

Western Equine Encephalitis Virus in Saskatchewan Garter Snakes and Leopard Frogs

Abstract. *Western equine encephalitis virus was isolated from two naturally infected snakes on first bleeding and from seven others at subsequent bleedings, both with and without preliminary chilling. One snake, with neither detectable virus nor serum neutralizing antibodies when first bled, developed viremia later. Viremia in garter snakes has a cyclic rhythm independent of the temperature of the environment. Virus was isolated from 6 frogs, and 50 out of 179 had detectable serum neutralizing antibodies. Infections with this virus are widely distributed in garter snakes and leopard frogs in the agricultural area of Saskatchewan.*

It has been demonstrated (*1–3*) that common garter snakes (genus *Thamnophis*) are susceptible to Western equine encephalitis (WEE) virus by both inoculation and mosquito bite and that they may serve as possible overwintering hosts of this virus. From 1961 to 1963 Spalatin *et al.* (*4*) found three species of garter snakes in Saskatchewan with serum neutralizing (SN) antibodies to WEE virus. At about the same time the virus was isolated in Utah (*5*) from four species of naturally infected snakes, including garter snakes; in these snakes viremia recurred in response to a lowering of environmental temperature. Thus the snakes could qualify as overwintering hosts of WEE virus in a temperate climate.

In Saskatchewan, frogs and garter snakes occupy the same habitats during summer months, mosquitoes are known to feed on both (*2, 3, 5, 6*), and both can be infected by the oral route (*4*). Since frogs form a large part of the natural diet of garter snakes, it seemed reasonable to investigate further the possible role of frogs in the epidemiology of WEE. Spalatin *et al.* found evidence of natural infections in *Rana pipiens* by demonstrating SN antibodies in the blood, but viremia was not detected (*4*). This report is concerned with observations on three species of garter snakes: *Thamnophis radix haydeni*, the Western plains garter snake; *T. sirtalis parietalis*, the red-sided garter snake; and *T. ordinoides vagrans*, the Great Basin garter snake; and one species of frog, the American leopard frog *Rana pipiens*.

Snakes and frogs were collected by hand in the field, shipped to the laboratory, and bled as soon after their arrival as possible. Frogs were usually bled the same day, but some snakes were not bled until 42 days after capture, during which time they were held at room temperature. Snakes were bled by inserting a fine capillary pipette behind the eye; frogs were pithed and then bled from the heart with the use of a 1-ml hypodermic syringe and 25-gauge needle. From 0.2 to 1.5 ml of blood could be obtained at each bleeding, depending on the size of the snake or frog.

The same procedures for virus and antibody studies were used on both snake and frog blood. For attempting virus isolation, sufficient blood was added to the diluent to make a 1 : 5 or 1 : 10 dilution, and the test animals were chicks up to 12 hours old and 10-day chicken embryos in groups of five to ten. The virus diluent contained, per milliliter: penicillin, 500 units; streptomycin, 2.5 mg; and heparin, 20 units; normal rabbit serum was added to give a concentration of 10 to 15 percent. Chicks were inoculated intraperitoneally or subcutaneously, and embryos allantoically, with 0.1 ml of inoculum. Adult mice and mice 3 to 5 days old, used for confirming isolations, were injected intracerebrally and intraperitoneally, respectively, with 0.03 ml of inoculum. Blood samples were either examined at the time of bleeding or stored at −20°C until they were tested. Serum obtained from each bleeding was also stored and, at the time of testing, was diluted as required. Virus isolates from snakes and frogs were identified by means of immune serum from rabbits that had been inoculated with WEE virus 85 (Rocky Mountain Laboratory) and mosquito isolate 1540–63 identified as WEE. Ten-day chicken embryos were used for testing serum samples for SN antibodies against known strains of WEE virus. Varying amounts of virus, diluted with nutrient broth containing antibiotics and 10 percent normal rabbit serum, were added to constant amounts of test serum and then incubated at 37°C for 2 hours before the eggs were injected. Normal rabbit serum was also used as the control for calculating neutralizing indices by the Reed-Muench method (*7*).