cells (lane A2) or induced cells containing the vector alone (lane C2). These data strongly suggest that the 23K protein is the product of the expression of the v-*ras*^{H} gene in *E. coli*.

Since the bacterially synthesized *ras* gene product appeared very similar to the Ha-MuSV p21^{ras}, we analyzed it for the biochemical properties of the viral protein. The bacterial p21^{ras} protein possesses the ability to autophosphorylate in the presence of guanosine triphosphate (GTP) (Fig. 3a, lanes C1 to C3). No such activity was found in extracts of *E. coli* cells containing the pJL6 vector alone (Fig. 3a, lanes A1 to A3) or uninduced cells containing the plasmid pJL*c*II*ras*1. Extracts of induced cells containing pJL*c*II*ras*1 also showed a guanosine diphosphate (GDP)–binding activity (Fig. 3b) that was not present in extracts of uninduced cells or in cells containing just the plasmid pJL6. Thus the bacterial p21 possesses both of the best characterized biochemical properties of Ha-MuSV p21.

Although previous observations (*6*) that both guanine nucleotide binding and autophosphorylation activity copurify with p21 suggest that these activities are present in that protein, they do not formally rule out the possibility that they reside on another protein, possibly one that binds p21. Our finding that the bacterially synthesized *ras* gene product also has these activities, however, confirms that these properties are intrinsic to the p21 produced from this gene. Observations (*13*) that *E. coli* has only low levels of protein kinases make it extremely unlikely that the kinase activity we observed is due to a bacterial protein. The Rous sarcoma virus p60^{src} also shows protein kinase activity when expressed in *E. coli* (*14*). These observations also indicate the enzymatic activity seen in virus-infected cells is an intrinsic property of the protein coded for by the viral oncogene.

Our observation that at least some bacterial p21 is soluble under nondenaturing conditions indicates that the bacterial strains expressing this protein should provide a convenient source for the preparation of large quantities of native protein for further study. The system described here also should allow the study of mutant p21s since *ras* sequences can be modified by site-specific mutagenesis and reintroduced into the expression vector. Both normal and mutant proteins may be characterized biochemically and analyzed for activity on biological systems. For example, it should be possible to determine the ability of each of these proteins to transform cells into which they are introduced by microinjection. Thus the expression system described above should facilitate biochemical and in vitro genetic approaches to our understanding of how Ha-MuSV p21^{ras} transforms cells.

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A Silent, Neutral Substitution Detected by Reverse-Phase High-Performance Liquid Chromatography: Hemoglobin Beirut

Abstract. *A substitution of alanine for valine at position 126 in the* β*-chain of hemoglobin was discovered in a hematologically normal adult male of Lebanese extraction. The variant* β*-globin was initially observed and subsequently purified by reverse-phase high-performance liquid chromatography (HPLC). Reverse-phase HPLC was also used to isolate the variant tryptic peptide of* β*-T13 that has alanine replacing valine at residue 126. The discovery of hemoglobin Beirut illustrates the usefulness of reverse-phase HPLC for the detection of neutral amino acid substitutions in proteins. The ability to detect neutral substitutions in undigested proteins is pertinent to the monitoring of genetic variation in human populations.*

Most of the hemoglobin variants that have been reported represent amino acid substitutions resulting in a charge change that alters electrophoretic mobility (*1*). However, on the assumption that nucleotide substitutions in the globin genes occur at random, a preponderance of neutral substitutions would be expected. The paucity of neutral variants discovered to date reflects at least in part the difficulty of detecting neutral substitutions electrophoretically. Reverse-phase high-performance liquid chromatography (HPLC) is a powerful tool for the separation of proteins and peptides (*2*). The globin chains of human adult (*3, 4*) and fetal (*5, 6*) hemoglobins are well resolved by this technique. Because protein separation by reverse-phase HPLC is based on differences in hydrophobicity, this technique was suggested as a means of detecting neutral amino acid substitutions in human hemoglobins and other proteins (*4*). We describe a neutral amino acid substitution in the β chain of human hemoglobin that was initially detected and subsequently purified by reverse-phase HPLC. A variant tryptic peptide was also isolated by reverse-phase HPLC.

In the course of a study of hemoglobin synthesis in cultures of erythroid progenitors, an abnormal peak was detected in a reverse-phase separation of globin chains prepared from mature erythroid colonies. The donor was a healthy adult male of Lebanese origin. The same HPLC pattern was subsequently observed in globin chains prepared from peripheral red blood cells of the same individual. The variant peak was also detected in the mother and sister of the donor. None of the three individuals was anemic or exhibited any abnormal hematological features. Red blood cell O_2 binding was normal. Hemoglobin analysis by standard electrophoresis on cellulose acetate or citrate agar, by isoelectric focusing in a 6 to 8 *p*H gradient, or by anion (*7*) or cation (*8*) exchange HPLC revealed no abnormal hemoglobins. The isopropanol test (*9*) for hemoglobin stability also gave normal results.

A hemolyzate was prepared from peripheral blood of the donor, and globin chains were separated by a modification of the reverse-phase HPLC method of Congote *et al.* (*5*). Reverse-phase HPLC of globin chains (Fig. 1) revealed an abnormal peak that eluted 1.8 minutes

earlier than β^A globin. The combined absorbance of β^A and the abnormal globin equaled the absorbance of α-globin at 214 nm, suggesting that the abnormal peak represented a variant β-globin (β^X). The variant β^X accounted for 44 percent of total β-globin in the donor, his mother, and sister (range 42 to 46 percent).

For structural studies, β-globin chains derived from 1.5 mg of hemoglobin were obtained by reverse-phase HPLC under the chromatography conditions of Fig. 1. Purified β^A and β^X globins were obtained by chromatography of the respective fractions under the same conditions. Tryptic peptides prepared from β^A and β^X were separated by reverse-phase HPLC (Fig. 2). The variant globin yielded an abnormal peak (β-Tx) that was eluted at 20.8 minutes. A decrease in the height of the peak that eluted at 23.0 minutes, in the expected position of β-T13 and β-T14, was also observed. Amino acid analysis of β-Tx gave the following composition, with the expected number of residues for β-T13 given in parentheses: Asx, 0.3 (0); Thr, 0.9 (1); Ser, 0.1 (0); Glx, 3.1 (3); Pro, 2.0 (2); Gly, 0.3 (0); Ala, 2.9 (2); Val, 0.2 (1); Leu, 0.1 (0); Tyr, 0.9 (1); Phe, 0.9 (1); His, 0.2 (0); Lys, 1.3 (1); and Arg, 0.1 (0) (*10*). The amino acid composition suggested that the peptide corresponded to β-T13 with valine replaced by alanine at residue 126. The assignment of glutamate or glutamine residues and the alanine substitution in β-Tx were confirmed by amino acid sequence analysis (*11*). The sequence Glu-Phe-Thr-Pro-Pro-*Ala*-Gln-Ala-Ala-Tyr-Gln-Lys, which represents residues 121 to 132, was obtained. The substitution of alanine for valine is consistent with the relative reverse-phase elution position, based on hydrophobicity (*12*), of both the variant tryptic peptide and the intact β^X globin.

The absence of hematological abnormalities in individuals with the hemoglobin Beirut variant is not surprising. Valine at residue 126 occupies a surface crevice of the H α-helix and is not involved in interchain or heme bonding (*13*). The reduced amount of hemoglobin Beirut relative to hemoglobin A could be due either to slightly decreased stability, not detectable with the isopropanol test, or to altered gene expression during transcription, RNA processing, or translation (*14*). The only other human β chain variant resulting from a substitution at residue 126 is hemoglobin Hofu (Val→Glu) (*15*), which was detected by electrophoresis. Hemoglobin Hofu comprised 50.6 percent of the hemolyzate and was not associated with clinical symptoms (*15*). The stability and O_2 binding of this variant were not reported.

Whereas position 126 is valine in the β chain and the structurally related δ chain of higher primates and most species, alanine is present at residue 126 in the hemoglobin β chain of the mouse, potoroo, and echidna (*16*), in the δ chain of the owl monkey (*16*), and as a variant in the δ chain of the gorilla (*17*). Other neutral amino acids have also been reported at residue 126 in other species (*18*). In view of the species variability at residue 126 of both the β and δ chains, hemoglobin Beirut might represent a neutral polymorphism in some human populations. Silent polymorphisms in which a neutral amino acid is substituted for another neutral amino acid undoubt-

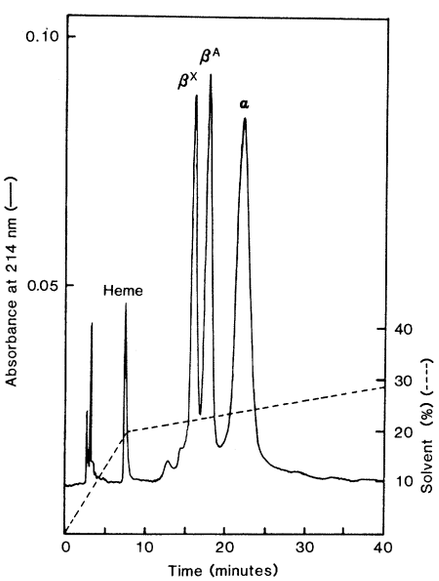

Fig. 1 (left). Detection of a β-globin variant by reverse-phase HPLC. Blood from a Lebanese adult male was drawn into acid-citrate-dextrose as anticoagulant. From a hemolyzate, 20 μg of hemoglobin was acidified with trifluoroacetic acid (TFA) (final concentration, 1 percent; weight to volume) and immediately injected onto a μBondapak C_{18} reverse-phase column (3.9 by 300 mm). Globin chains were separated by a modification of the method of Congote *et al.* (*5*). The column, maintained at 40°C, was equilibrated with solvent A, consisting of a solution of acetonitrile and water (40:60) to which was added 0.2 percent TFA (weight to volume). Globin chains were eluted with linear gradient segments of solvent B, consisting of a solution of acetonitrile and water (60:40) to which was added 0.2 percent TFA (weight to volume) as follows: 0 percent B to 20 percent B in 8 minutes, then to 29 percent B in 32 minutes. The effluent was monitored at 214 nm. After the globin chains were separated, the column was flushed with a linear gradient to 100 percent B in 5 minutes, then acetonitrile for 5 minutes, and finally 100 percent A for 15 minutes to reequilibrate the column. The flow rate was 1 ml/min. The column was stored in acetonitrile.

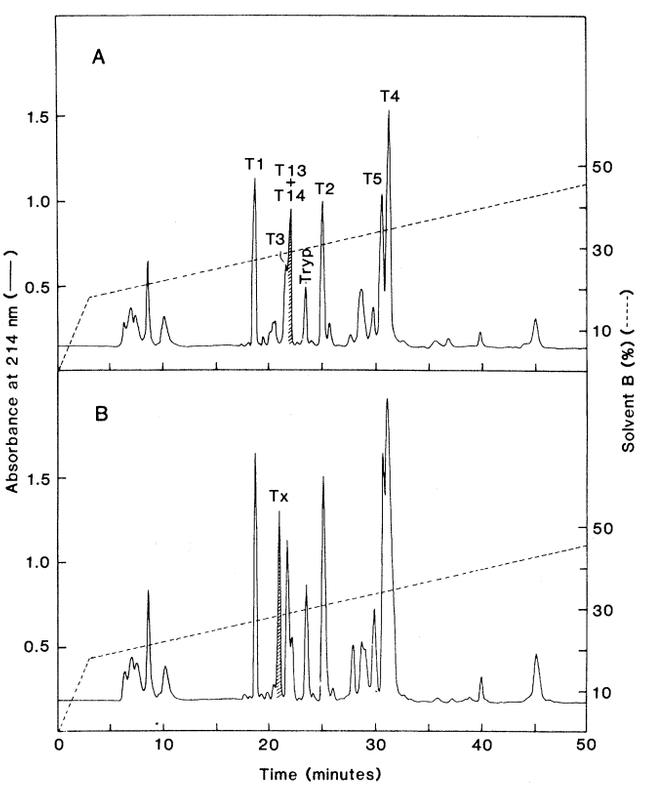

Fig. 2 (right). Reverse-phase HPLC separation of tryptic peptides of β^A and β^X. The variant and normal β chains were isolated directly from a hemolyzate (1.5 mg of hemoglobin) by reverse-phase HPLC as described in Fig. 1, except that detection was done at 280 nm. Individual β^X and β^A globins were collected, diluted with an equal volume of H_2O, and rechromatographed. The purified β^X and β^A globins (12 to 15 nmole) were lyophilized and digested with trypsin (TPCK, Miles) (*22*) for 20 hours. Tryptic peptides were separated by reverse-phase HPLC on a μBondapak C_{18} column operated at room temperature. The column was equilibrated with solvent A (0.1 percent TFA in H_2O, weight to volume). Elution of peptides was achieved with linear gradient segments of solvent B (0.05 percent TFA in acetonitrile, weight to volume) as follows: 0 percent B to 18 percent B in 3 minutes, then to 46 percent B in 47 minutes, at a flow rate of 0.6 ml/min. The labeled peaks designate tryptic peptides from the β chain of hemoglobin. These fractions were collected and identified by amino acid analysis. Sequence analysis (*11*) of T13 + T14 and of Tx was performed. (A) Tryptic peptides from 12 nmole of β^A; *Tryp*, trypsin. (B) Tryptic peptides from 15 nmole of β^X.

edly exist, as illustrated by the discovery of either isoleucine or threonine at position 75 in human $^{A}\gamma$-globin (*19*). Also a silent polymorphism involving a neutral amino acid substitution (valine or isoleucine) at residue 31 of human skeletal muscle carbonic anhydrase (CA III) has been reported (*20*); this was detected by reverse-phase HPLC separation of tryptic peptides of CA III from pooled muscle samples. Silent substitutions in hemoglobin may also be common in the baboon, as illustrated by the description of hemoglobin Papio B (*4*).

The ability to detect amino acid substitutions in proteins has provided a biochemical means of estimating the amount of genetic variation in human populations and the frequency of mutations (*21*). The screening procedures used for these estimates include electrophoresis and measurements of enzyme activity and protein stability. The discovery of hemoglobin Beirut illustrates the potential of reverse-phase HPLC for the detection of neutral amino acid substitutions in proteins. Since the frequency of neutral amino acid substitutions could be two to three times greater than the frequency of charge substitutions, reverse-phase HPLC increases our ability to detect genetic variations in proteins and to estimate the frequency with which mutation results in a previously cryptic type of protein variation.

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An Inverted Repeat Borders a Fivefold Amplification in Satellite DNA

Abstract. *One variant of a complex satellite DNA of the Bermuda land crab is significantly longer than the average repeat unit of the satellite. The extra DNA in the variant is accounted for by a fivefold tandem amplification of a 0.142-kilobase sequence. The amplified sequence is bounded by a tetranucleotide inverted repeat; the upstream arm of the inverted repeat is missing from two other variants of the satellite. The latter variants contain only one copy of a sequence that is closely related to the amplified sequence. By contrast, in several satellite DNA's of other organisms, extra DNA is inserted.*

Bermuda land crab DNA contains two satellites. One of them has a guanine and cytosine (G+C) content of 63 percent, a density in CsCl gradients of 1.721 g/cm^3, and comprises 3 percent of the total DNA (*1*). When products of an Eco RI digest of that satellite were inserted into the Eco RI site of plasmid pBR322 and amplified in *Escherichia coli* strain HB101, 13 of the 16 clones recovered had inserts 2.06 to 2.09 kilobases (kb) long, similar to the sizes of repeat units

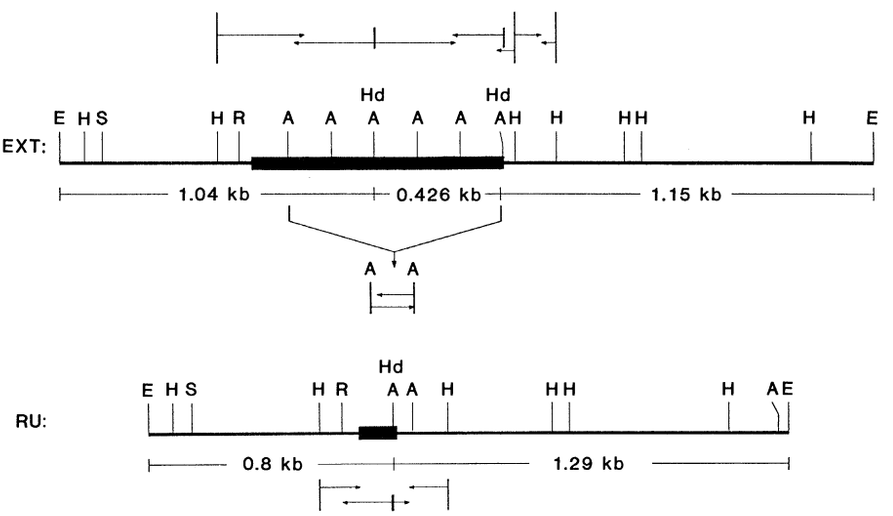

Fig. 1. Restriction maps of EXT and RU showing sites for Alu I (*A*), Eco RI (*E*), Hind III (*Hd*), Hinf I (*H*), Rsa I (*R*), and Sau3A I (*S*). The amplified (six copies) of the 0.142-kb repeats in EXT and the homologous 0.127-kb sequence in RU are indicated by filled boxes. The Hind III fragments seen in Fig. 2, A and B, are shown below each map. Fragments were sequenced by the Maxam-Gilbert procedure (*7*). (i), Sequencing strategies for the Hinf I 0.989-kb and the 0.136-kb fragments of EXT are shown above its restriction map; the strategy for a mixture of five 0.142-kb repeats resulting from digestion of the 0.989-kb fragment with Alu I is below. (ii) One of several sequencing strategies for Hinf I fragments of RU that contain the homolog to the amplified sequence is shown below the restriction map of RU; sequencing was also done with Hpa II fragments (*5*). Vertical bars that terminate arrows indicate the 5′ ends of fragments that were labeled with ^{32}P; the length of arrows indicates the distance read. Labeled fragments were either strand-separated or cut with restriction enzymes at the sites shown.