standing of the molecular action of diphtheria toxin.

Note added in proof: After communication of our results R. Delange (*20*) has reinvestigated the amino acid sequence of fragment A in the region corresponding to residues 171–174 (Fig. 1) where our sequences had differed in the position of a Ser residue. He now finds the sequence (Ser)-Val-Glu-Tyr in agreement with our data, that is, the originally published amino acid sequence (*4*) has to be corrected in this position.

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High-Level Expression in *Escherichia coli* of Enzymatically Active Harvey Murine Sarcoma Virus p21^{ras} Protein

Abstract. *The gene for the Harvey murine sarcoma virus (Ha-MuSV) p21*^{ras} *protein was fused to the amino-terminal portion of the bacteriophage* λ c*II gene on the expression vector pJL6. The fusion was such that transcription was controlled by the well-regulated phage* λ p_L *promoter, and translation initiated in the* c*II gene continued in frame into the* ras *gene sequences that code for p21. When the* p_L *promoter was derepressed, the* Escherichia coli *cells harboring the fusion plasmid synthesized 23,000-dalton protein, which represented more than 10 percent of the total cellular protein. This protein was chimeric and contained 14 residues, which were specified by the vector; these residues were followed by all of the amino acids that make up Ha-MuSV p21*^{ras} *except for four residues at the amino-terminal end. The protein appears similar to Ha-MuSV p21*^{ras} *in that it undergoes immunoprecipitation by monoclonal antibodies directed toward that protein, binds guanosine diphosphate, and is capable of autophosphorylation.*

The Harvey murine sarcoma virus (Ha-MuSV) is a replication-defective retrovirus capable of transforming cultured fibroblasts in vitro and inducing solid tumors in animals (*1*). It was isolated from solid tumors that developed in rats that had been injected with Moloney murine leukemia virus (Mo-MuLV). The transforming activity of Ha-MuSV is due to the presence of the v-*ras*^{H} oncogene (*2*), which codes for a 21,000-dalton (21K) protein called p21^{ras} (*3*). The *ras* gene of Ha-MuSV is closely related to sequences in the mouse and rat genomes that are called c-*ras* (*4*). It is likely that Ha-MuSV arose by recombination between rodent c-*ras* sequences and Mo-MuLV.

While the means by which p21^{ras} transforms cells remains unknown, it has several interesting properties. This protein binds guanine nucleotides (*5*) and is capable of autophosphorylation (*6*). Further study of the properties of this protein would be greatly facilitated by the availability of methods to prepare it in large quantities. One approach that has been applied to the protein products of several other viruses would be to produce the protein in bacteria. We chose to express the Ha-MuSV p21^{ras} by placing it in the plasmid pJL6 (*7*). This plasmid contains the 13 amino-terminal codons of the bacteriophage λ *c*II gene placed under the transcriptional control of the well-regulated phage λ p_L promoter.

pJL6 contains a single Hind III site adjacent to the Cla I site at the end of the *c*II gene on this plasmid. Translation beginning at the *c*II gene initiation codon should continue past this Hind III site further into the pBR322-derived sequences. This enzyme cleaves between the first and second base of a codon within the open reading frame defined by that translation start point. The sequence of the v-*ras*^{H} gene (*8*) predicts that Hind III also cuts that gene between the first and second base of a codon. Therefore, it was possible to create an in-frame fusion between the *c*II and *ras* genes by ligating a 880-bp Hind III fragment from plasmid H1 (*9*) containing most of the *ras* gene to Hind III–cleaved pJL6 DNA. A plasmid having the *c*II and *ras* genes in the same orientation was designated pJL*c*II*ras*1 and a plasmid in which the genes were in opposite orientations was called pJL*c*II*sar*1 (Fig. 1).

Plasmid pJL*c*II*ras*1 was introduced into *Escherichia coli* N4830 (*10*). This strain contains a highly deleted λ prophage carrying the mutant *c*I857 tem-

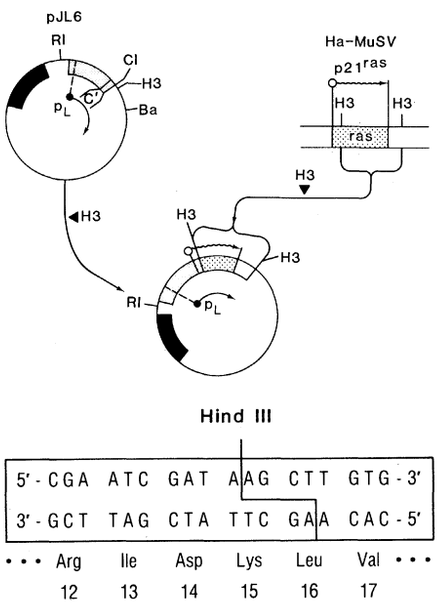

Fig. 1. Construction of pJL*c*II*ras*1. A 880-bp Hind III fragment [corresponding to positions 4040 to 4920, as shown by Ellis *et al.* (*2*)] containing most of the Ha-MuSV *ras* gene was isolated from plasmid H1 (*9*) and inserted into the Hind III site of pJL6 so as to generate pJL*c*II*ras*1. Symbols: solid segments, ampicillin resistance genes; heavily dotted segments, DNA derived from phage λ; and finely dotted segments, coding region for the Ha-MuSV p21^{ras} protein. The sequence shown is that of the *c*II-*ras* junction.

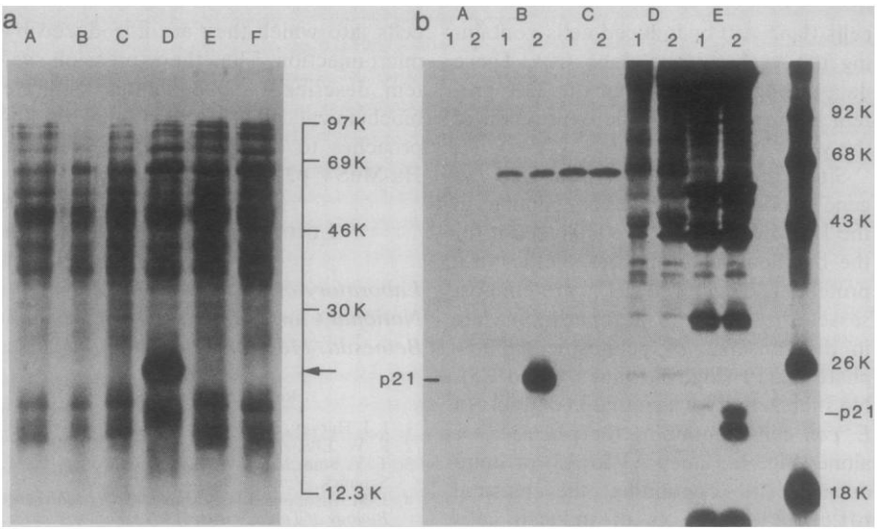

Fig. 2. Expression of the v-*ras*^{H} gene in *E. coli*. (a) Radiolabeling of bacterial cells. *Escherichia coli* cells were grown at 32°C, induced by shifting the temperature to 41°C, labeled with [^{35}S]cysteine, and lysed (*7*). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography. (Lane A) Uninduced *E. coli* N4830[pJL*c*II-*sar*1] cells; (lane B) induced N4830[pJL*c*II-*sar*1] cells; (lane C) uninduced N4830[pJL*c*II-*ras*1] cells; (lane D), induced N4830[pJL*c*II-*ras*1] cells; (lane E) uninduced N4830[pJL6] cells; (lane F) induced N4830[pJL6] cells. The arrow indicates the migration position of the p21 protein produced in *E. coli*. (b) Immunoprecipitation of bacterial p21 from *E. coli* strains containing the v-*ras*^{H} gene. Extracts of labeled bacterial cells (induced and uninduced) were prepared as described above and immunoprecipitated. The immunoprecipitated proteins were resolved by SDS-PAGE and visualized by autoradiography. The NRK and Ha-NRK tissue culture cells were labeled with [^{35}S]methionine. Extracts were from (A) uninduced *E. coli* N4830[pJL*c*II*ras*1] cells; (B) induced N4830[pJL*c*II*ras*1] cells; (C) induced N4830[pJL6] cells; (D) NRK cells; (E) Ha-NRK cells. Serums used for the immunoprecipitations were, in the lanes marked 1, normal rat serum; and in the lanes marked 2, monoclonal antibody Y13-259 against p21 (*15*).

perature-sensitive repressor. At 32°C the repressor is active and the p_L promoter on the plasmid is repressed. At 41°C the repressor is inactive and the p_L promoter is induced, which allows high-level expression of genes under its transcriptional control. These regulatory controls have been observed for the phage λ *c*II protein produced from the pKC30*c*II parent of pJL6 (*11*) and for a protein resulting from a fusion of the *c*II gene with the avian myelocytomatosis virus (MC29) v-*myc* gene (*7*).

The lysogen carrying the *c*II-*ras* gene fusion plasmid was grown at 32°C and bacterial proteins were labeled with [^{35}S]cystine before and after induction at 41°C. Cells induced for 15 minutes synthesized large amounts of a 23K protein (Fig. 2a, lane D) not observed in uninduced cells (Fig. 2a, lane C). Furthermore, this protein is not observed in uninduced or induced cells containing the pJL6 vector alone (Fig. 2a, lanes E and F) or in uninduced or induced cells containing the v-*ras* insert in the incorrect orientation (Fig. 2, lanes A and B). Induced cells were lysed in the presence of lysozyme and a mixture of NP40 and sodium deoxycholate (*12*), and the extract was centrifuged. Proteins in both the supernatant and pellet fractions were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue staining (data not shown). Most of the 23K protein was found in the pellet fraction although a significant amount of it could be identified in the supernatant fraction. This protein was not observed in either fraction for uninduced cells containing the pJL*c*II*ras*1 plasmid or induced cells containing the pJL6 vector without an insert. The 23K protein was the major protein present in the pellet of the induced cells containing the v-*ras*^{H} gene and constituted more than 10 percent of the total cell protein.

The size of the 23K protein agrees well with that predicted for the expected *c*II-*ras* fusion protein (22.5K). This protein should be identical to the Ha-MuSV p21^{ras} except for the substitution of its four amino terminal residues with 14 amino acids derived from the phage λ *c*II gene and plasmid pBR322 sequences on the pJL6 vector. This protein was similar to authentic Ha-MuSV p21^{ras}, in that it was immunoprecipitated by a monoclonal antibody specific for that protein (Fig. 2b, lane B2). This 23K protein was the only protein in extracts of induced cells containing the pJL*c*II*ras*1 plasmid that was precipitated by this antibody. This protein was not precipitated by normal rat serum (lane B1) and was not precipitated in extracts of uninduced

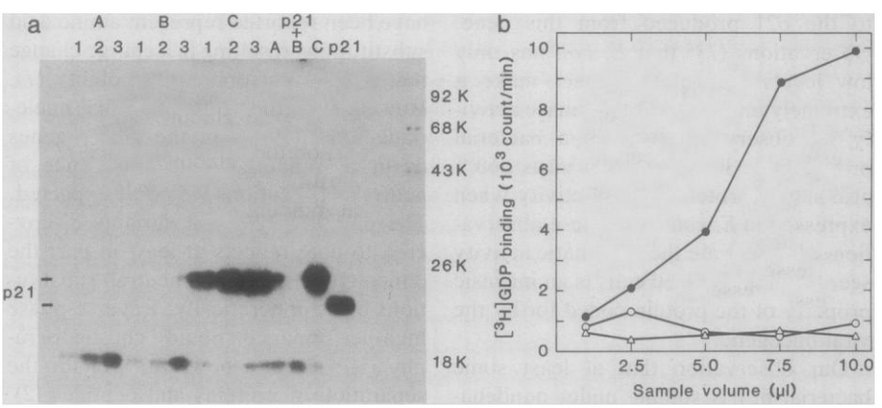

Fig. 3. (a) Autophosphorylation of bacterial p21. Supernatant fractions from *E. coli* cells lysed by lysozyme NP40 and sodium deoxycholate (*12*) were immunoprecipitated with a monoclonal antibody Y13-259 against p21 (*15*); 50 μl of buffer (0.05*M* tris-HCl, *p*H 8.0, 5 m*M* $MgCl_2$, 0.1*M* NaCl) and 25 μCi of [α-^{32}P]GTP (400 Ci/mmole) was added to each pellet. After incubation at 37°C for 30 minutes, the pellet was washed several times with buffer, and proteins were resolved by electrophoresis (SDS-PAGE). The extracts used were from (A) induced *E. coli* N4830[pJL6] cells; (B) uninduced N4830[pJL*c*II*ras*1] cells; (lane C), induced N4830[pJL*c*II*ras*1] cells. In A, B, and C, 2 μl of viral p21 purified from Ha-MuSV-transformed NIH3T3 cells was added to 10 μl of supernatant fraction of bacterial extract prior to immunoprecipitation. The last p21 lane is the 2 μl of purified viral p21 autophosphorylated without immunoprecipitation. The volumes of bacterial extracts used were: Lanes 1, 2 μl; lanes 2, 10 μl; lanes 3, 50 μl. (b) [^{3}H]GDP-binding activity of *E. coli* p21. Supernatant fractions of *E. coli* cells prepared as described above were incubated with 150 μCi of [^{3}H]GDP (13 Ci/mmole) and immunoprecipitated with the Y13-238 monoclonal antibody to p21 (*15*). After the pellets were washed several times with buffer (0.05*M* tris-HCl, *p*H 8.0, 0.1*M* NaCl, 1 percent NP40), the GDP-protein complexes were collected on Millipore filters and the radioactivity was counted. The extracts used were from (△) induced N4830[pJL6] cells; (○) uninduced N4830[pJL*c*II*ras*1] cells; and (●) induced N4830[pJL*c*II*ras*1] cells.

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