Table 1. Antigenic relationship of HER virus and rat virus in hemagglutination-inhibition (HI) tests.

Serum from rabbit immunized with	Titer against 6 HA units of	
	R1SR3 TC1	3PRE 308
HER agent, R1SR3	160	160
Rat virus, 3 PRE 308	80	160
Rat virus, H-1	<20	<20
Normal rat brain	<20	<20
Before immunization	<20	<20

1.39 g/cm^3. These particles were about 20 mμ in diameter, somewhat angular in outline, and occasionally appeared to have knob-like subunits on their surface (see arrow in Fig. 7). No other virus-like particles were seen in these preparations. Assays of dialyzed fractions in suckling rats revealed that most of the infectivity was associated with the fractions containing most of the particles. These results are consistent with the conclusion that the biological activity (production of hemorrhagic encephalopathy) of these suspensions is associated with a small dense virus physically similar to those previously described (*4*).

Limited trials have shown that the HER agent (infected rat brain or rat embryo tissue culture fluid) agglutinates red blood cells from guinea pigs but not those from chicken, sheep, rhesus monkey, or man (type O). Our first tests indicate that serum from rabbits immunized with HER virus will inhibit hemagglutination. Furthermore, hemagglutination-inhibition tests (Table 1) with rat virus antigen and antiserum (provided by L. Kilham) indicate a definite antigenic relationship to strain 3 PRE 308 but not to the strain H-1 of rat virus (*4*).

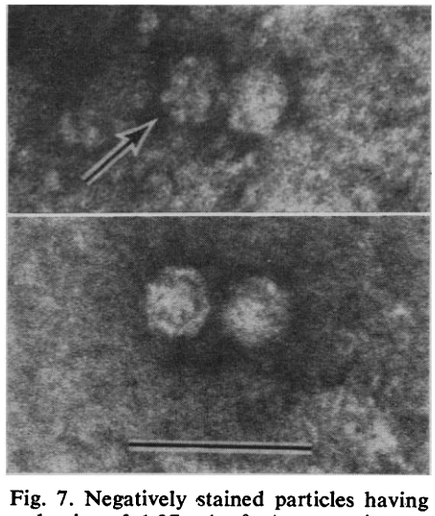

Fig. 7. Negatively stained particles having a density of 1.37 g/cm^3. Arrow points to knob-like subunits on the surface of one particle (bar = 50 mμ).

Hemorrhagic encephalitis in infected rats has been seen on rare occasions by Kilham and Margolis (*5*) during their extensive work with rat virus (*4*), although we have not found any other reports of a virus which attacks the central nervous system in the manner described above (*6*). Together with our experimental results this suggests that we have isolated a strain of rat virus with biological properties quite different from those of strains previously isolated. We propose HER virus as a tentative name for our isolates and suggest that they be called the HER strains when they can be assigned a definitive taxonomic classification.

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Suppressor Selection for Amino Acid Replacements Expected on the Basis of the Genetic Code

Abstract. *In studies with A protein mutants of the tryptophan synthetase of* Escherichia coli, *missense supressors have been used to select for codon changes and corresponding amino acid replacements that are normally unobservable. The technique has permitted the detection of additional amino acid replacements expected on the basis of the genetic code.*

Extensive genetic and biochemical studies with the A-gene–A-protein system (tryptophan synthetase) of *Escherichia coli* K-12 have resulted in the identification of many single amino acid substitutions associated with single mutational events (*1, 2*). By examining the amino acid changes associated with mutations from prototroph to auxotroph to prototroph, multiple amino acid substitutions at the same position in the A protein have been detected. In all but one case, the codons for the particular amino acids in each substitution are related in that a single nucleotide change in an RNA codon can account for the change. For example, mutant A46, a strain in which there is an amino acid change from glycine to glutamic acid at position 210 in the A protein, yields revertants containing either glycine, alanine, or valine at this position (Fig. 1). From the RNA codons shown in Fig. 2 (*3*) it is apparent that single nucleotide changes in the A46 glutamic acid codon, which is probably GAA (*4*), should also result in the replacement of glutamic acid by aspartic acid, lysine, and glutamine (*1, 2*). The failure to find these substitutions in the A protein of the revert-

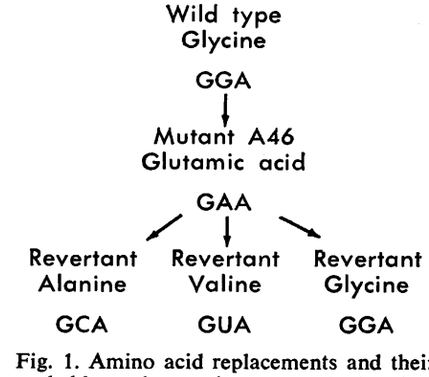

Fig. 1. Amino acid replacements and their probable codon assignments at the A46 site (position 210) in the A protein of tryptophan synthetase (*1, 2*).

ants of mutant A46 has been explained on the assumption that these changes do not result in prototrophy; that is, an A protein with aspartic acid, lysine, or glutamine at position 210 is enzymatically inactive.

The availability of several specific missense suppressor genes has suggested one possible experimental approach to the verification of this conclusion. The rationale for these experiments was that, if one mutant codon were converted to a second mutant codon, the change might be detected if a missense suppressor specific for the second mutant codon were present. If the second mutant codon were read incorrectly by the suppressor, resulting in the occasional insertion of an amino acid which restores enzymatic activity, the codon change should be detected by the ability of such a strain to grow on glucose-salts medium. Using this approach we have been able to select for a codon change with mutant A46 which results in an amino acid substitution of glutamic acid by aspartic acid at position 210 in the A protein, and, with mutant A33, we have selected a codon change which results in the change from methionine to valine at position 48 in the A protein.

The change from wild type to mutant at the A46 site results from substitution of glutamic acid for glycine (Fig. 1). Selection for the mutant-to-mutant change from glutamic to aspartic acid was attempted with a missense suppressor, su58+ (*5*), of the A58 missense mutation (Table 1). This suppressor does not suppress mutant A46 but restores partial prototrophy to mutant A58. Since the mutational change from wild type to mutant A58 results in a substitution of glycine by aspartic acid at position 233 in the A protein (*2*), it seems likely that su58+ leads to the occasional insertion of glycine in response to the aspartic acid codon corresponding to position 233. We reasoned that the amino acid substitution of glutamic by aspartic acid at the A46 site could be detected in cells containing the A58 suppressor, since, in the presence of su58+, glycine would be occasionally incorporated at position 210, with resultant production of some enzymatically active (glycine-containing) A protein molecules.

A stock containing the A46 alteration and the suppressor gene su58+ was prepared by transduction techniques with bacteriophage Plkc (*6*).

Table 1. Selection procedure for obtaining a change from glutamic to aspartic acid at the A46 site (position 210) using the su58+ gene.

Strain	Steps	Phenotype	Amino acid and probable codon at position 210	
A46		Auxotroph	Glu	GAA
	← Introduce su58+			
A46 su58+		Auxotroph	Glu	GAA
	← Select prototrophs			
$A46^{Asp}$ su58+		Prototroph	Asp*	GAU or GAC
	← Replace su58+ with su58−			
$A46^{Asp}$		Auxotroph	Asp	GAU or GAC

* Some of the A protein molecules probably have glycine inserted at position 210 as a consequence of the presence of the su58+ gene.

This stock was tryptophan-requiring, an indication that gene su58+ does not suppress mutant A46. The A46 su58+ stock was examined for revertants according to the methods of Allen and Yanofsky (*7*). Thirty slow-growing colonies were isolated, and one was found by genetic tests to be a suppressed mutant. The A gene was removed from the su58+ background by transduction and yielded a tryptophan auxotroph. This auxotroph was examined in genetic tests, and it did not yield wild-type recombinants when crossed by mutant A46, an indication that auxotrophy was due to a genetic change at or near the A46 site in the gene. Transduction experiments demonstrated that the auxotroph was suppressed by the su58+ suppressor gene. This new mutant was designated A46-X.

The procedures used in the isolation of the A protein of strain A46-X were the same as those described for the wild-type A protein (*8*). Since genetic tests indicated that the site of the A46-X alteration was at or near the site of the A46 mutation, a chymotryptic peptide that normally contains position 210 was selected for isolation. This peptide, CP2 (containing residues

—U—		—C—		—A—		—G—	
UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC		UCC		UAC		UGC	
UUA	Leu	UCA	Ser	UAA	Ochre	UGA	?
UUG		UCG		UAG	Amber	UGG	Trp
CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
CUC		CCC		CAC		CGC	
CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CUG		CCG		CAG		CGG	
AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
AUC		ACC		AAC		AGC	
AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
AUG	Met	ACG		AAG		AGG	
GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
GUC		GCC		GAC		GGC	
GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GUG		GCG		GAG		GGG	

Fig. 2. Nucleotide sequences of RNA codons and corresponding amino acids. The horizontal lines separate codons with different first nucleotides. Each vertical column contains codons with the same middle nucleotide.

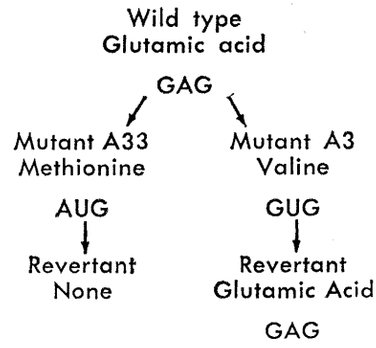

Fig. 3. The A3 and A33 amino acid changes at position 48 in the A protein and the probable corresponding codon assignments (*11*).

203 to 211), was isolated from peptide patterns prepared by two-dimensional chromatography, and electrophoresis at *p*H 3.7, as described (*9*). Table 2 shows the amino acid composition of the peptide from the A proteins of mutant A46-X, mutant A46, and wild-type. The A46-X peptide differs from the A46 peptide by a change from glutamic acid to aspartic acid or asparagine. The intact A46-X peptide moved toward the anode on electrophoresis at *p*H 6.5, showing that the amino acid change was to aspartic acid rather than to asparagine. The new mutant A46-X is designated A46^{Asp}.

The finding of the amino acid substitution of glutamic by aspartic acid, resulting from a single mutational event at the A46 site, is consistent with there being a single nucleotide difference between the RNA codons for glutamic and aspartic acids (Fig. 2). This finding also explains the observation that revertants of mutant A46 do not contain aspartic acid at position 210. Furthermore, this result indicates that the A58 suppressor gene, su58+, is not allele-specific but probably can suppress the same codon at any position, since the same suppressor is active on two sites separated by 22 codons, both specifying aspartic acid.

Table 2. Amino acid composition of peptide CP2. The compositions are given as the molar ratios of constituent amino acids based on the average value for a single residue determined from the total composition of the peptide.

Amino acids	Molar ratios		
	Wild type	A46	A46-X
Asp	1	1	2.0
Glu	1	2	1.3
Pro	2	2	1.9
Gly	1	0	0
Ala	2	2	2.0
Leu	1	1	0.98
Phe	1	1	0.82
Amino acid replacement		Gly ↓ Glu	Glu ↓ Asp

Since mutants A58 and A46^{Asp} are sensitive to the same suppressor, we studied the effectiveness of the su58+ gene on each of the mutants and on a double-mutant strain containing both the A46^{Asp} and the A58 alterations. The A proteins of the various suppressed and unsuppressed strains were examined for enzymatic activity in the indole to tryptophan and indoleglycerol-phosphate to tryptophan reactions. As shown in Table 3, the A proteins of mutant A46^{Asp} and A58 have no activity in the conversion of indoleglycerol phosphate to tryptophan, while low-level activity is restored in these strains by the su58+ gene. This low-level activity is undoubtedly responsible for the ability of the suppressed mutants to grow on glucose-salts medium. Furthermore, the level of activity restored in strains A58 su58+ and A46^{Asp} su58+ is nearly identical, an indication that the missense suppressor functions equally well in misreading the two nonallelic aspartic acid codons. In addition, a strain carrying mutational alterations A46^{Asp}, A58, and the su58+ gene, did not grow in glucose-salts medium and did not have detectable amounts of indoleglycerol phosphate to tryptophan activity. This finding is compatable with the notion that the suppressor acts independently on each of the mutant codons so that the amount of activity restored would be equal to the product of the extent of suppression at each site (*10*) or approximately 0.01 to 0.02 percent. Finally, aspartic acid represents the ninth amino acid that has been detected at position 210 in the A protein.

Since A46^{Asp} is auxotrophic, reversion studies can be performed with this mutant to determine which of the seven possible amino acid substitutions predicted on the basis of the RNA codons in Fig. 2 can be found by single nucleotide changes from the aspartic acid codon.

Mutant A33 is unique in being the only known CRM+, A-protein mutant that does not revert (*11*). It has a methionine residue at position 48, a position occupied by glutamic acid in the wild-type A protein (Fig. 3). The change from glutamic acid to methionine can only be explained by a two-nucleotide change in an existing glutamic acid codon. The fact that mutant A33 does not yield either full or partial revertants suggests that none of the codons derivable by a single nucleotide change from the AUG methionine codon specifying position 48 will restore prototrophy. Since the A3 mutation resulting in a change from glutamic acid to valine at position 48 (Fig. 3) is suppressed by the su3+ gene, we reasoned that, using this suppressor, it might be possible to select for a conversion of the A33 methionine codon (AUG) to a valine codon (GUG), a single nucleotide change.

Table 3. Enzymatic activities of the A proteins in mutants A46^{Asp}, A58, and the double mutant A46^{Asp}-A58 in the presence and absence of su58+. A, units per milliliter of enzymatic activity in the conversion of indole to tryptophan; B, units per milliliter of enzymatic activity in the conversion of indoleglycerol phosphate to tryptophan.

Strain	A*	B*	B/A (%)
Wild type	102	49	48
A46^{Asp}	670	0	
A46^{Asp}su58+	322	4.3	1.3
A58	650	0	
A58su58+	490	5.7	1.2
A46^{Asp} A58	470	0	
A46^{Asp} A58su58+	342	.25	.07

* Assayed in the presence of excess B protein. One unit of activity is defined as the amount of enzyme required to convert 0.1 μmole of substrate in 20 minutes at 37°C.

By techniques similar to those described, the su3+ gene was introduced into mutant A33. This strain was tryptophan dependent. When cells of this strain were plated for revertants, one class of slow-growing colonies appeared. One of these colonies was purified and examined by transduction experiments. An A gene auxotroph designated A33-X was obtained by removal of the su3+

Table 4. Amino acid composition of peptide TP15.

Amino acids	Molar ratios		
	Wild type	A33	A33-X
Arg	1	1	0.96
Asp	5	5	4.8
Thr	3	3	2.7
Ser	1	1	1.1
Glu	3	2	2.5
Pro	3	3	2.7
Gly	3	3	3.0
Ala	5	5	5.0
Val	0	0	1.3
Met	0	1	0
Ile	4	4	4.1
Leu	5	5	4.8
Phe	1	1	0.92
Amino acid replacement		Glu ↓ Met	Met ↓ Val

suppressor. This strain did not yield recombinants when crossed with the original A33 mutant. However, it differed from A33 in being sensitive to suppression by su3+ and in being revertible.

Tryptic peptide TP15, the peptide that contains the amino acid substituted by the A33 change, was isolated by column chromatography on Dowex 1-X2 resin (*12*). This peptide is one of the last eluted by this technique, appearing when the concentration of the eluting buffer is approximately 2*N* with respect to acetic acid. The peptide was further purified by paper electrophoresis at *p*H 3.7 prior to analysis of the amino acids. Table 4 shows the amino acid composition of TP15 from wild-type, mutant A33, and from mutant A33-X. It is evident that the su3+ gene selected the change from a methionine codon to a valine codon at position 48.

The ability to select for this change from methionine to valine as a single mutational event provides in vivo support for assigning AUG as the RNA codeword for methionine and GUG as a codeword for valine.

In the presence of two distinct suppressor genes we have been able to isolate CRM+ A mutants which have amino acid replacements in their A proteins predicted by the genetic code, but which are not found by ordinary reversion analysis because the amino acid replacements do not restore prototrophy. The new change in mutant A46 brings the number of amino acids that replace glutamic acid to four, out of a possible six predicted on the basis of the RNA codons.

As additional CRM+ mutants and suppressor genes are characterized, it will be possible to use our technique to extend the analysis of mutational relationships predicted by the genetic code.

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4. Abbreviations used are: A, adenine; G, guanine; C, cytosine; U, uridine; CRM+, mutants which produce an A protein which reacts with antibodies prepared with wild-type A protein (cross-reacting material); Arg, arginine; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Trp, tryptophan; Cys, cysteine; Lys, lysine; Tyr, tyrosine; and His, histidine.
5. We use su+ to denote the active form of the two suppressor genes in our experiments.
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Hemoglobin $\alpha_2\beta_2^{121\ Lys}$ Chemical Identification in an Egyptian Family

Abstract. *Study of the amino acid composition and amino-terminal residue in the abnormal peptide of an electrophoretically slow hemoglobin revealed that the globin's structure was* $\alpha_2\beta_2^{121\ Lys}$ *(Oβ). This hemoglobin had been provisionally classified as Hb E or as a new hemoglobin. The clinical significance of this abnormality is not yet defined.*

We have chemically identified a hemoglobin variant located in the Nile Delta of Egypt, United Arab Republic. At *p*H 8.7 in starch-gel electrophoresis, this hemoglobin (Hb) moved to the zone of Hb E/A_2 and was tentatively classified as Hb E. However, a suggestion was made that the abnormal hemoglobin might be a new one because, unlike Hb E, the molecule was distinguishable from Hb A_2 in two-dimensional starch-gel electrophoresis (*1*).

The propositus and his family possessed the abnormality in a heterozygous form. The slow component was separated from hemoglobin A on starch gels and was eluted and purified (*2*). Polypeptide-chain hybridization with canine hemoglobin and with known abnormal human hemoglobin showed that the molecular abnormality

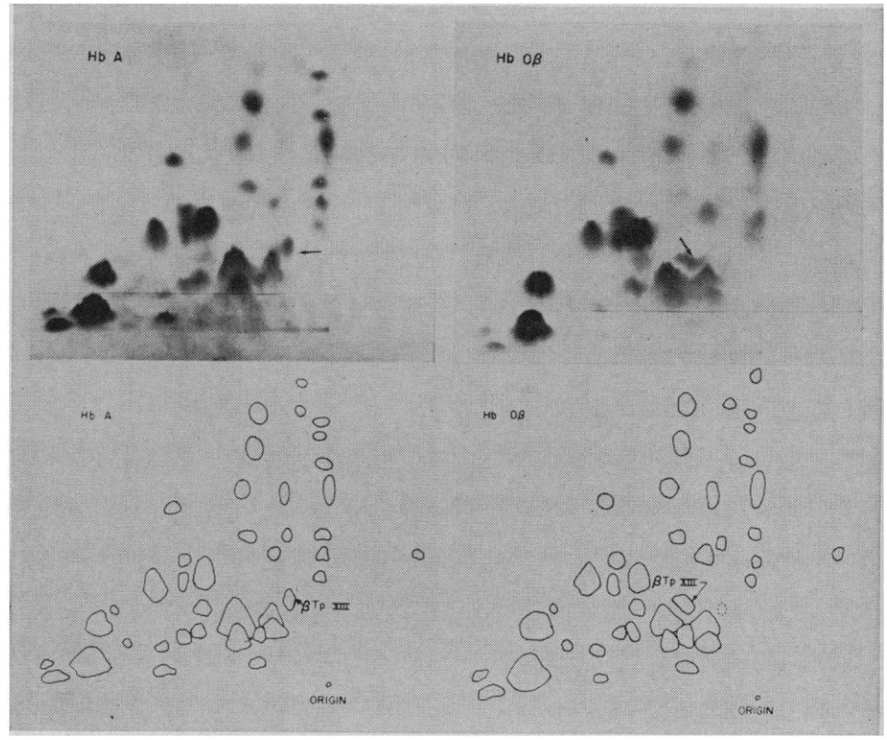

Fig. 1. Fingerprints and corresponding diagrams of trypsin digests of hemoglobins A and $\alpha_2\beta_2^{121\ Lys}$ (Oβ). Peptide β Tp XIII has moved farther toward the cathode on the left in the abnormal hemoglobin.