CRYPTIC MUTANTS OF BACTERIOPHAGE T4

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THE apparent mutation rate depends upon a number of contingencies: the primary error rate, the probability of repair, error avoidance by the apparatus of DNA replication, and the probability of detection. The probability of detection of those base pair substitutions which produce an amino acid substitution appears to be highly variable, and presumably reflects the sensitivity of polypeptide functions to small variations in primary structure.

Fine-scale mapping has often revealed a highly nonrandom distribution of mutations among sites. In the T4*rII* system, for instance, 809 of the first 1609 independently isolated mutants were observed to map into only two sites (BENZER 1961). Genetic data indicate that the number of *rII* sites capable of mutation by base pair substitution is about 1700 (EDGAR *et al.* 1962; STAHL, EDGAR and STEINBERG 1964), and this estimate is supported by quasi-chemical measurements (GOLDBERG 1966). When frameshift mutations are excluded from consideration, however, fewer than 3% of these potential sites have been identified (FREESE 1959; BENZER 1961; ORGEL and BRENNER 1961; see Chapter 5 of DRAKE 1970). What then is the nature of the apparently immutable sites?

Two possible answers deserve serious consideration: many sites are extremely weakly mutable, or else many amino acid substitutions are undetected in standard screening systems because they fail to exert significantly deleterious effects upon protein function. The first possibility is supported by the observation that T4*rII* amber and ochre mutations, which are likely to be detected with uniformly high efficiencies, nevertheless appear at very different frequencies at different sites during the course of hydroxylamine mutagenesis (BRENNER, STRETTON and KAPLAN 1965). The second possibility is supported by the observation that a disproportionately large fraction of T4*rII* base pair substitution mutants contain amber codons, compared to the fraction expected from random base pair substitutions (BENZER and CHAMPE 1961; CHAMPE and BENZER 1962). The second possibility is also supported by the observation that approximately one-quarter of the beginning of the *rIIB* cistron is dispensable (DRAKE 1963). Furthermore, the *rII* function is overproduced by the (possibly trivial) criteria of the standard screening system, since many *rII* ochre mutants are well suppressed even though the efficiency of chain propagation is well below 10% (BRENNER and BECKWITH 1965).

We have approached the problem of the missing sites by designing procedures to increase the efficiency of detection of leaky mutants. It was already clear that temperature-sensitive (*ts*) mutations of the *rII* region are readily obtained

(DRAKE and MCGUIRE 1967a,b; DRAKE, unpublished results), and nineteen such mutations are sufficiently inactive at 42°C to be mapped into sites. In the *rII* system, the wild-type plaque is relatively small with a fuzzy edge, whereas the mutant plaque is large with a sharp edge. The *ts* mutants of this system frequently produce semi-*r* plaques at low temperatures. We supposed that these partially defective mutants might be rendered fully defective by secondary amino acid substitutions which would otherwise be innocuous. Nitrous acid was adopted as a mutagen, first because it induces both transitions (see Chapter 13 of DRAKE 1970), and second because the mutations which it induces show relatively strong clustering into sites (BENZER 1961; KRIEG 1963), so that new sites should be easily recognized. It will be convenient to introduce two terms to describe mutations at these new sites. A leaky mutation which is used to enhance the recovery of normally undetectable mutants will be called a *sensitizing mutation.* (Our sensitizing mutations contain the designation *SM*. This is fortuitous, the symbol merely indicating the spontaneous origin of the mutation during the storage of a T4 stock.) The mutations which are exposed by a sensitizing mutation, and which are otherwise poorly detectable, will be called *cryptic mutations.*

MATERIALS AND METHODS

All experiments were carried out using *Escherichia coli* and bacteriophage T4B. Media, procedures for growing stocks, selection of mutants, scoring of phenotypes, and genetic crosses have all been described previously (BENZER 1961; EDGAR *et al.* 1962; DRAKE and MCGUIRE 1967a,b). Plaque morphology was scored on B cells, and ability to grow in a lambda lysogen was scored using OP or KB cells. Stocks were grown on BB cells, which do not discriminate between *rII* mutants and the wild type.

Nitrous acid mutagenesis was performed by exposing stocks to 0.08 or 0.09 M $NaNO_2$ in 0.2 M sodium acetate buffer at p.H 4.0 at room temperature. The reaction was terminated by a 10-fold dilution into 1.0 M Tris buffer at pH 8.0. Mutagenesis of T4*rSM104* was performed by exposing the stock to approximately 20 lethal hits using 0.08 M $NaNO_2$. The treated particles were immediately adsorbed to BB cells at an average of eight particles per cell for a single cycle of growth in liquid culture. The resulting average burst size of two indicated the occurrence of strong multiplicity reactivation. The progeny particles were plated on B cells, and after 18 hr at 30°C an *r* mutant frequency of 0.7% was observed, whereupon mutants were collected. Under these conditions the selection of more than one member of a clone will occur very rarely. The spontaneous background in the untreated stock was 0.025%, or 3.6% of the induced mutant frequency. Control experiments at lower levels of inactivation, and without a cycle of growth in BB cells, indicated that about 2% to 3% of *r* mutants should have been observed. Multiplicity reactivation therefore appears to select against the induced mutants, a result also observed after ultraviolet mutagenesis (DRAKE, unpublished observation). Mutagenesis of T4*rSM122* was performed by exposing the stock to approximately 6.5 lethal hits using 0.09 M $NaNO_2$. In this case the particles were plated directly on B cells. The *r* mutant frequency was 1.0%, compared to a spontaneous background of 0.05% (5% of the induced level).

Complementation tests were performed by adsorbing an average of three particles of each parental type to OP cells in L broth, and completing lysis at 40 min with chloroform. The test was performed at 37°C unless a *ts* mutant was involved, in which case it was performed at 41°C.

RESULTS

T4 wild type produces plaques on the order of 1.5 mm diameter on B cells and on OP cells. Its *rII* mutants, if fully defective, produce plaques on the order of

3 mm diameter on B cells, and these plaques exhibit much sharper edges than do r^+ plaques because of the absence of lysis inhibition. No plaques are produced on OP cells. A continuous range of intermediate types of *r* mutants can also be obtained. These typically produce intermediate plaque morphologies on B cells, and may produce small plaques on OP cells. The wild-type plaque morphology on B cells is mutationally altered much more readily than is the ability to grow on lambda lysogens, and many mutants which produce *r* plaques on B cells also produce wild-type plaques on OP cells. The two sensitizing mutants which we have studied produce plaques on B cells at 32°C which are larger and less turbid than are r^+ plaques, but which are clearly distinct from the *r* plaques produced by fully defective mutants.

A collection of mutants exhibiting much more of the mutant character than did the parental type was collected after nitrous acid mutagenesis of two strains carrying sensitizing mutations. A few derivatives of *rSM122* were collected which were not completely defective, but which were obviously more defective than the parental type. These mutants produced small plaques on OP cells at 32°C, but were unable to produce visible plaques at 37°C. (Both *rSM104* and *rSM122* produce nearly normal plaques on OP cells at 37°C.) Of 390 *r* mutants obtained from *rSM104*, 168 or 43% were classified as *rII*. Of 342 *r* mutants obtained from *rSM122*, 154 or 45% were classified as *rII*. These frequencies are typical of *r* mutants produced by base pair substitutions.

The two sets of mutants were mapped into segments and then into sites using recombination spot tests and the mapping deletions of BENZER (1961). (If a newly induced mutation were cryptic, then deletion mapping would often produce ambiguous results. We therefore constructed deletion-plus-sensitizer double mutants for use in many of the mapping experiments; see Figure 2.) The resulting mutational spectra, together with BENZER's (1961) spectrum of mutations obtained by nitrous acid mutagenesis of the wild type, appear in Figure 1. The horizontal line segments refer to map segments defined by deletions, and are well ordered. However, segments containing no mutants are not included in these maps. The heavy line segments identify the regions containing the sensitizing mutations, A6c for *rSM104* and A2g for *rSM122*. The *A* cistron is on the left, and the *B* cistron is on the right. When sites on our two spectra are aligned vertically, they are identical. The sites *r131*, *rN24* and *r117* from the BENZER map are also known to coincide with the corresponding sites on our maps, but the rest of the alignment between our mutants and the BENZER set is conjectural. Sites are not ordered within a segment. Each independently arising mutant is represented by a box, the filled boxes representing the cryptic mutants to be described below. Some cryptic mutants could not be unequivocally assigned to a particular segment, but could be assigned to a group of segments. These sites appear on line segments floating above the main axis of the map.

Few deletions appeared in either set of *rII* mutants. The set obtained from *rSM104* contained three unequivocal multisite mutations, while the set obtained from *rSM122* contained only one. A number of mutants produced stocks containing fewer than 10^{-8} revertants, but their mutations all behaved like point

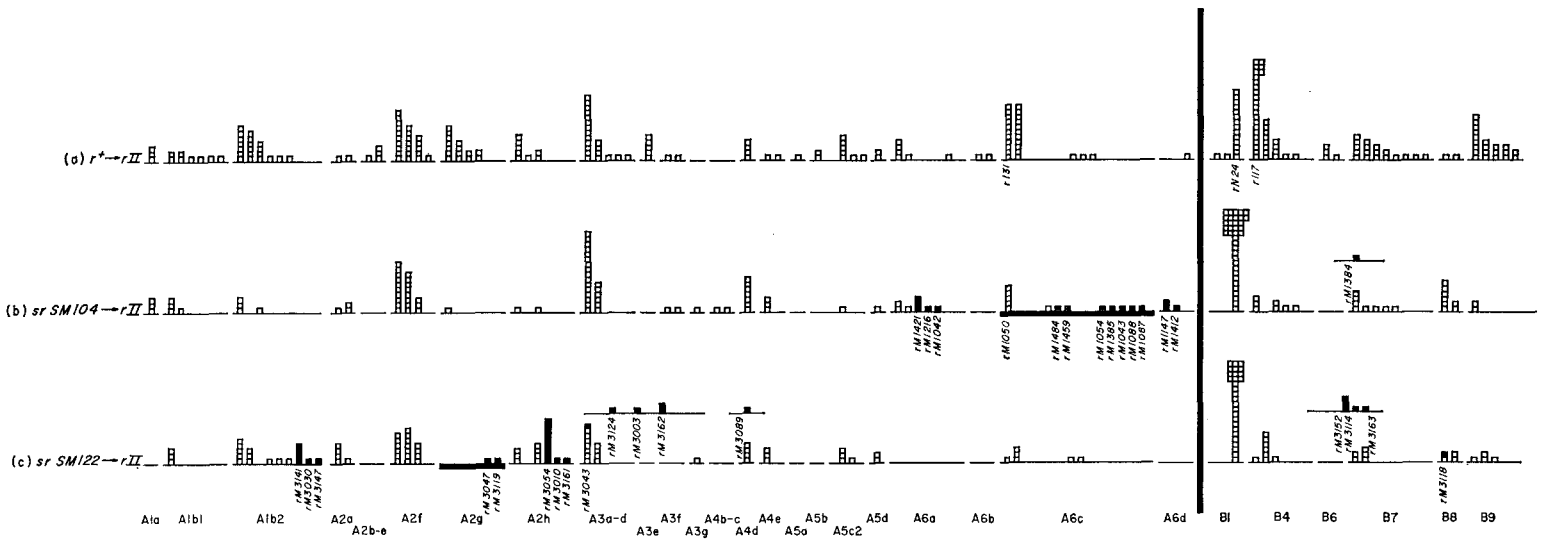


FIGURE 1.—Mutational spectra produced by nitrous acid mutagenesis of T4B (top, from BENZER 1961) and two of its semi-*r* derivatives. The *A* cistron is on the left of the vertical bar, and the *B* cistron is on the right. Solid squares represent cryptic mutants. The sensitizing mutations fall in the semgents denoted by heavy horizontal lines. Segments defined by the standard mapping deletions are identified at the bottom of the Figure. "The three dotted boxes in segment A6c of the middle spectrum represent putative but unconfirmed cryptic mutants."

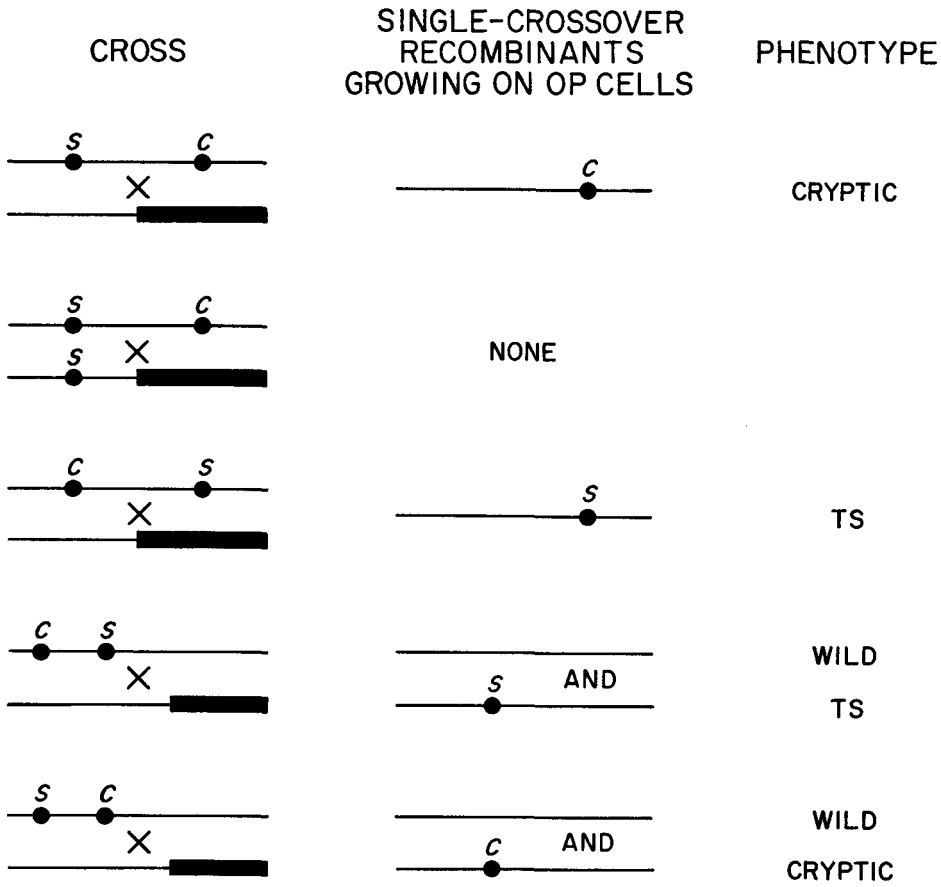
lesions in mapping experiments, and could contain at most only very short deletions. The overall large deletion frequency was therefore 1.25%. Since about 10% of most collections of spontaneous *rII* mutants contain deletions, correcting our observed frequency for the spontaneous background indicates that only about 0.8% of the induced mutants contain deletions. This frequency is much lower than the 8% observed by TESSMAN (1962) in similar experiments.

The two spectra obtained by us are very similar except for cryptic mutants, and are also similar to the 260-mutant spectrum obtained by BENZER. Certain hot spots (sites containing many independently arising mutations) which appeared in the BENZER spectrum in segments A2g, A3e and A6c, however, seem not to have appeared in our spectra, and quantitative differences have appeared among the three largest hot spots. The hot spots *r131* and *r117*, which account for 50% of all spontaneous *rII* point mutations and contain frameshift mutations (DRAKE and MCGUIRE 1967a,b), are relatively more strongly represented in the 1961 spectrum, which also clearly contained a much higher frequency of mutants from the spontaneous background than do either of the present collections. The *rN24* site, which is strongly induced by all mutagens which generate transitions, is much more weakly represented in the 1961 spectrum than in the new spectra. It should be noted that the conditions of mutagenesis used by BENZER (1.8 M $NaNO_2$, pH approximately 6.5) are very different from those employed by us.

The *rSM104* spectrum contains a much higher density of sites in the A6 region than does either of the other two spectra. These sites, and many sites from the *rSM122* spectrum within segments A1, A2 and A3, frequently contained cryptic mutations. Mutants were classified as cryptic if they produced recombinants able to grow on OP cells in crosses with a mapping deletion, but not in crosses with the same mapping deletion coupled to the sensitizing mutation (Figure 2). When the appropriate deletion mutant was not available for this test, the mutant was crossed against deletions which approached the mutant pair (sensitizer plus putative cryptic) from one or both sides, and 10 to 18 isolates able to grow on OP cells were isolated (Figure 2). Each isolate was examined for its plaque morphology on B cells, and was also crossed with the appropriate sensitizing mutation in order to attempt the reconstruction of the fully defective double mutant. The results of these tests are summarized in Table 1 as well as in Figure 1. Three mutants from the *rSM104* spectrum could not be unequivocally analyzed in any of these tests. These mutants are likely to contain cryptic mutations because they map in a region relatively devoid of base-pair substitution sites, but they must be considered "unconfirmed" at the present time.

A considerable number of crosses was performed during the characterization of the cryptic mutations within the A6 segment of the *rSM104* spectrum. The data are summarized in Figure 3. Analysis of the map distances by means of the data of STAHL, EDGAR and STEINBERG (1964) and of GOLDBERG (1966) suggests that the interval surrounding *rSM104* in which cryptic mutations are exposed corresponds to 130 to 170 amino acids. The cryptic mutations are approximately evenly distributed throughout this interval.

Limited tests were performed of the specificity of interaction between cryptic



FIGUER 2.—Tests to identify and isolate cryptic mutants. In cases where the position of the putative cryptic site was not known relative to the deletion terminus, multiple tests were performed. The "cryptic" phenotype consists either of a semi-*r* plaque morphology (with or without temperature sensitivity), or of the ability to reform a fully mutant derivative when recombined with a sensitizing mutation.

and sensitizing mutations. The cryptic mutation *rM1366* (separated from *rSM104*, and mapping at the *rM1421* site) was crossed against *rSM122*, and the cryptic mutation *rM3054* (separated from *rSM122*) was crossed against *rSM104*. Approximately 3500 progeny from each cross were screened for the fully defective *r* plaque morphology on B cells at 30°C. No *r* isolates were observed which contained the appropriate double mutant configuration. The interaction between sensitizing mutation and cryptic mutation therefore appears to be allele-specific.

We have determined the plaque morphologies of nine isolated cryptic mutants from each spectrum. These are arranged downwards in Table 2 in approximate order of increasing defectiveness. Their behavior depends upon temperature. On B cells, several mutants appear wild type at 32°C but produce *r* plaques at 43°C. On OP cells, nearly all mutants grow at 43°C, but many produce tiny

TABLE 1

Identification of cryptic mutants

Method of identification	Sensitizer	
	rSM104	*rSM122*
Crosses with mapping deletion ± sensitizer*	*M1042*	*M3003*
	M1216	*M3030*
	M1384	*M3043*
	M1421	*M3089*
		M3114
		M3118
		M3124
		M3141
		M3147
		M3152
		M3162
		M3163
Splitting and reconstruction of double mutant and/or analysis of phenotype	*M1043*	*M3010*
	M1087	*M3047*
	M1088	*M3054*
	M1147	*M3119*
	M1385	*M3161*
	M1412	
Unconfirmed (see text)	*M1054*	
	M1459	
	M1484	

* *M3030*, *M3141* and *M3147* were identified by their ability to recombine with the deletion *r1241* (which covers *rSM122* but not the cryptic site) and with the deletion *r1364* (which covers the cryptic site but not *rSM122*), but not with the deletion *rEM66* (which covers both sites). This test is the diagnostic equivalent of the "deletion ± sensitizer" test.

plaques. There is a slight tendency for the cryptic modifiers of *rSM122* to be less defective than the cryptic modifiers of *rSM104*. This difference probably results from the deliberate selection of obviously leaky double mutants from the *rSM122* stock, but not from the *rSM104* stock.

Despite the fact that the sensitizing mutations are located in the *A* cistron, both spectra contain cryptic mutants which map in the *B* cistron (Figure 1). This result, together with the observation of intracistronic complementation between *rIIA* mutants (CHAMPE and BENZER, described in FINCHMAM 1966), suggested the existence of complex quaternary interactions among the *A*-cistron and *B*-cistron polypeptides. We sought further evidence for such interactions by means of two tests, intracistronic complementation among *B* mutants and mutual suppression between *A* and *B* mutants. Eighteen slightly leaky or temperature-sensitive *rIIB* mutants were tested for complementation in a total of 39 pairwise combinations. These mutants were scattered throughout the *B* cistron. No evidence for complementation was observed, the largest factor of increase over either single-parent control being less than two-fold. CHAMPE and BENZER, on the other hand, observed increases of more than 400-fold with pairs of *A*-cistron

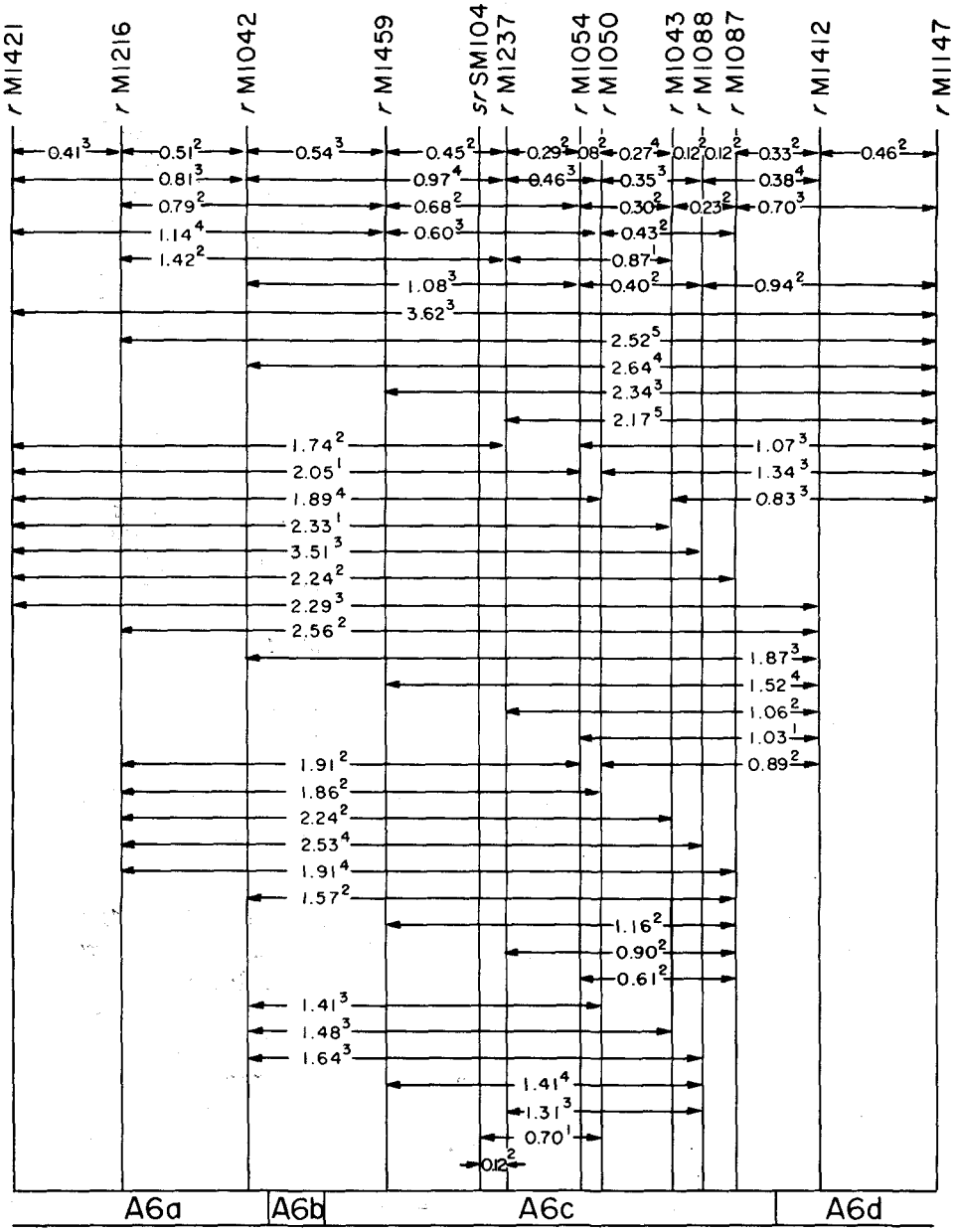


FIGURE 3.—Map of the cryptic mutants exposed by *rSM104*. Map segments defined by standard deletions are indicated at the bottom. Distances represent twice the mean percentage of wild-type recombinants, and superscripts represent the number of independent crosses performed. The mutants *rM1050* and *rM1237* are not cryptic. The unconfirmed cryptic mutant *rM1484* and the cryptic mutant *rM1385* were not mapped in these experiments. The cryptic mutant *rM1384* falls in the *B* cistron.

TABLE 2

Plaque morphology of cryptic mutants in the absence of the sensitizing mutation

Mutant	Appearance on B cells				Appearance on OP cells	
	30°C	32°C	37°C	43°C	30°–37°C	43°C
rM3124	w	w	sr	r	w	w
rM3162	w	w	sr	r	w	w
rM1385	w	w	fr	r	w	w
rM3054	w	w	sr	r	w	ti
rM3113	w	w	sr	r	w	ti
rM3161	w	sr	r	r	w	w
rM1421	sr	sr	fr	r	w	w
rM3152	sr	sr	sr	r	w	ti
rM1147	sr	sr	r	r	w	w
rM1216	sr	sr	r	r	w	w
rM1087	fr	fr	r	r	w	w
rM3010	sr	fr	r	r	w	ti
rM3089	sr	fr	r	r	w	ti
rM1042	sr	r	r	r	w	ti
rM1043	fr	r	r	r	w	ti
rM3114	sr	fr	r	r	w	0
rM1412	fr	r	r	r	w	ti
rM1088	fr	r	r	r	w	0
rSM104	sr	sr	r	r	w	ti
rSM122	sr	sr	r	r	w	ti

w = wild type, maximum lysis inhibition.
sr = semi-*r*, some lysis inhibition, partially lysed halo-like edge.
fr = fuzzy-*r*, less lysis inhibition, but fuzzy edge.
r = fully mutant, no lysis inhibition, sharp edge.
ti = tiny plaque.
0 = no visible plaque.

mutants. In the second test, the reversion properties of 18 *A*-cistron mutants were examined. All of these mutants were missense, being reverted by base analogues and not suppressed by any of a set of suppressors of UAA, UAG and UGA codons. An average of four independent revertants of each mutant was selected, and each revertant was backcrossed to the wild type to identify suppressors mapping in the *B* cistron. None was found.

DISCUSSION

Although cryptic mutants are easily discovered, they usually would not be harvested during routine screening tests, first because many are insufficiently *r*-like at 32° or 37°C to attract the eye, and second because all grow well on lambda lysogens, at least at 37°C. Even when separated from the sensitizing mutation, however, all of the cryptic mutations studied are deleterious under at least some conditions. It is of course still possible that other sensitizing mutations might expose cryptic mutations which were indistinguishable from the wild type, or that some of the cryptic mutations which we did not separate from the sensitizing mutation would turn out to be similarly invisible.

About 6% to 8% of the isolates obtained from the *rSM104* background were cryptic, and 11% from the *rSM122* background were also cyptic. (The higher frequency from *rSM122* probably resulted from the deliberate selection of some mutants which were more *r*-like than the sensitizing mutant, but still were not fully mutant.) Our limited tests failed to indicate any synergism between a sensitizing mutation and a cryptic mutation isolated by means of a different sensitizing mutation. If this is generally the case, then a set of several dozen sensitizing mutations might well expose hundreds of different cryptic mutants. This procedure might be made even more efficacious by using mutagens which induce both transitions and transversions, and which exhibit less hot spotting than does nitrous acid. It is therefore reasonable to suppose that a large fraction of the unidentified T4*rII* base-pair substitution sites are not intrinsically immutable. This conclusion is supported by the observation that hot spotting is at least as intense among cryptic mutants as it is among ordinary mutants (Figure 1). A large fraction of amino acid substitutions in the *rII* polypeptides apparently fail to reduce gene function below what is required to pass through the standard screening tests. With the possible exception of the purple-adenine *ad3* mutants of Neurospora (DE SERRES, personal communication), this plasticity of primary structure seems to be rather widely observed. The underlying protein structural chemistry has been elegantly probed in studies of mutant vertebrate hemoglobins (PERUTZ *et al.* 1968; PERUTZ and LEHMANN 1968).

If cryptic base pair substitutions do in fact occur at typical rates at most sites, then most screening systems will tend to underestimate forward mutation rates. A reasonable estimate of the fraction of readily detected spontaneous T4*rII* mutations which contain base pair substitutions is 14% (FREESE 1959; DRAKE 1970). Since the fraction of detected base-pair substitution sites is below 3%, the true *rII* mutation rate is likely to be several-fold higher than previously estimated. It is also likely, however, that the *rII* region is somewhat less sensitive to base pair substitutions than is the average gene, since *rII* ochre mutants are more easily suppressed than are ochre mutants in many other cistrons. A reasonable corrected mutation rate for T4 would therefore be about 10^{-7} per base pair duplication, or about 10^{-4} per cistron duplication, or about 2% per chromosome duplication (DRAKE 1969).

We observed fewer than 1% deletions among induced mutants, whereas TESSMAN (1962) reported that 8% of *rII* mutants induced by nitrous acid contained large deletions. This large difference is not readily explicable on the basis of different experimental conditions. The TESSMAN mutants and the *rSM122* mutants were collected under similar or identical conditions of temperature (20°C *vs.* room temperature), pH (3.7 *vs.* 4.0), buffer, nitrite (0.2 M KNO_2 *vs.* 0.09 M $NaNO_2$), and survival. Some critical difference seems to have existed between the two experiments, however, for which we have no explanation. The differences between the BENZER (1961) and the present spectra, on the other hand, are very minor, and are largely explicable on the basis of a much larger contamination by the spontaneous background in the earlier collection.

One unexpected outcome of these experiments was the observation of muta-

tional synergism between lesions in the epistatic *A* and *B* cistrons. It therefore seems likely that the **A** and **B** polypeptides, encoded by the corresponding cistrons, interact rather directly. Since intracistronic complementation is demonstrable for *A* mutants but not for *B* mutants, and since this kind of complementation is also probably the result of subunit interactions in an oligomeric protein (FINCHAM 1966), it is likely that the minimum *rII* gene product consists of $\mathbf{A_2B}$. Neither the intercistronic nor the intracistronic mutational synergism is likely to become explicable in chemical terms until the three-dimensional structures of **A** and **B** are known. Other examples exist, however, of intracistronic interactions between moderately distant sites, one of the most impressive having been observed in the *E. coli* tryptophan synthetase A protein (YANOFSKY, HORN and THORPE 1964).

Many of these data were obtained by Miss LYNNE BARTENSTEIN, whose patient persistence has been crucial to the entire project. Some of the early mapping experiments were performed by Mr. ROBERT PASSOVOY. The work was supported by grant E59 from the American Cancer Society, grant GB6998 from the National Science Foundation, and grant AI-04886 from the National Institutes of Health, USPHS. R.E.K. was supported as a Predoctoral Trainee by USPHS Training Grant GM-510.

SUMMARY

About a tenth of the T4*rII* mutants induced by nitrous acid within a leaky *rII* background contain a second very leaky (cryptic) *rII* mutation which specifically interacts with the original (sensitizing) mutation to produce gene inactivation. The cryptic mutations usually map in the vicinity of the sensitizing mutation. However, they occasionally map in a different cistron altogether. Hot spotting is as prevalent among cryptic mutations as it is among ordinary base-pair substitution mutations. There is no need to suppose that as yet undetected base-pair substitution sites are particularly immutable. Instead, mutations at these sites probably escape detection because of the plasticity of composition of the corresponding polypeptide. In contrast to an earlier report, we detected hardly any deletions after nitrous acid mutagenesis. Although intracistronic complementation has been observed among *A* cistron mutants, we could detect none among *B* cistron mutants.

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