COMPLEMENTATION BETWEEN *ad-5/7* ALLELES IN YEAST

W. P. COSTELLO AND E. A. BEVAN[1]

Botany School, University of Oxford, Oxford, England

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INTRAGENIC complementation occurs when a full or partial restoration of nonmutant phenotype results from the association of two different mutant alleles in a heterozygote, heterocaryon, or heterogenote. It has been demonstrated for a number of gene loci in a variety of microorganisms which include fungi. (FINCHAM and PATEMAN 1957; GILES, PARTRIDGE and NELSON 1957; CATCHESIDE and OVERTON 1958), bacteria (HARTMAN, HARTMAN, SERMAN and LOPER 1958; MORSE 1962), and bacteriophage (CHAMPE and BENZER 1962). It has also been shown to occur in Drosophila (CARLSON 1959; WELSHONS and VON HALLE 1960; GREEN 1961; DORN and BURDICK 1962).

In 1958 CATCHESIDE and OVERTON showed that the pattern of relationships between complementing alleles could be recorded as a simple two-dimensional matrix and could be represented topographically as a simple straight line with distinct end points, which they called a complementation map. The relationship of alleles of most of the gene loci already studied can be represented in this manner. Recently, however, an increasing number of complementation maps have been reported which cannot be portrayed in this manner. Some of these are linear but do not possess end points (CARLSON 1961; CHAMPE and BENZER 1962; GROSS 1962; KAPULER and BERNSTEIN 1962), others are nonlinear (CATCHESIDE 1960; ISHIKAWA 1960, 1962a, 1962b; BERNSTEIN and MILLER 1961; BEVAN and WOODS 1962; SUYAMA and BONNER 1962; WOODWARD 1962). In this report, a further exception is described. Data are presented which show that the complementing *ad-7* mutant alleles of *Saccharomyces cerevisiae* may be mapped as a circle with a short tail segment.

ROMAN (1956a) was the first to isolate what he termed *ad-5* and *ad-7* mutant strains. These, when crossed, produced prototrophic diploids and he concluded that they were mutants of two different gene loci. He further concluded that these loci were closely linked since they were inseparable in 163 meiotic segregations. At the same time, ROMAN reported that mutation frequently affected both the *ad-5* and *ad-7* loci simultaneously to give what he termed *ad-5 ad-7* strains. (The symbol *ad* is used throughout the text to represent alleles conferring a growth requirement for adenine.) This finding has been confirmed both by DORFMAN (1963, 1964) and by the results of the present work. CATCHESIDE (1958) later interpreted this situation as a case of intragenic complementation, and MORTIMER (1963) states that he considers *ad-5* and *ad-7* alleles to be mutations of a single locus.

[1] Present address: Department of Botany, Queen Mary College, London, E.1, England.

If we are dealing here with a single locus, however, then our results indicate a situation unlike that encountered in studies with other gene loci. We have found that all *ad-5* alleles behave as a single complementation subunit: they never complement each other or *ad-5 ad-7* alleles, but do complement all *ad-7* alleles. Although the *ad-7* alleles fall into many complementation subunits, they also never complement any of the *ad-5 ad-7* alleles. It will have become clear in view of these remarks that the complementation map presented here is derived from the results of crosses of *ad-7* strains only, although exhaustive tests were made on the *ad-5* and *ad-5 ad-7* strains.

It should be stated that the classification of alleles into the three groups is dependent upon their complementation reactions with three standard tester strains, *ad-5*, *ad-7*, *ad-5 ad-7*, each of which so far has shown no intragroup complementation. To simplify expressions we have modified ROMAN's nomenclature and adopted the following terminology for the different types of mutant strains isolated:

ad-5/7	unclassified mutant alleles
ad-5 *ad-7*	mutant alleles classified by their complementation reaction with the standard tester strains
ad-5–7	mutant alleles showing no complementation with any of the standard tester strains

The information leading to the construction of our complementation map was derived by determining the phenotypes of diploids each heterozygous for a pair of independently isolated mutant alleles. To facilitate their isolation we used a selective technique which was a modification of that described by ROMAN (1956b). He made use of the fact that mutants of either *ad-1* or *ad-2* gene loci besides possessing a requirement for adenine, accumulate a red pigment. A strain mutant at either one of these loci and at any locus acting prior to them in adenine synthesis would therefore be white since the red pigment could not then be formed. ROMAN isolated such mutant white cells from sectors and papillae which arose spontaneously in aging red colonies. Further analysis of these white cells revealed mutants of four loci, including *ad-5/7* mutants.

Since we desired to obtain an extensive range of mutant alleles we modified ROMAN's technique in two ways. Firstly, we used two different mutagens, ultraviolet light (UV) and ethyl methanesulphonate (EMS). The latter is an alkylating agent whose potent mutagenic action has already been demonstrated using a variety of different organisms, including yeast (FAHMY and FAHMY 1956; WESTERGAARD 1957; LOVELESS 1958; HESLOT, FERRARY, LEVY and MONARD 1959; COSTELLO, BEVAN and MILLER 1963). Secondly, cells from whole white colonies as well as from sectors were isolated from platings of treated red *ad-2 cells*. In this way we probably obtained a higher proportion of the possible mutants which arose, and did not limit the spectrum to only those whose growth rate could compete successfully with parental *ad-2* cells, as ROMAN did.

We have presented our mutation data for two reasons. First, when compared with those of ROMAN (1956b), and DORFMAN (1963), who used ROMAN's isolation procedure, they illustrate how the frequency of mutation to different alleles may vary according to the techniques used for their isolation. Secondly, the data

on the frequencies of UV and EMS induced mutations as they affect each complementation subunit serve to emphasise how a complementation map may also vary according to the mutant isolation procedure.

MATERIALS AND METHODS

The parental *ad-2.0* strain used for the isolation of the mutants was separated from strain 1358 18d kindly supplied by DR. D. HAWTHORNE. The wild-type haploid strains used in crosses to separate the *ad-5/7* alleles from the mutant *ad-2.0*, *ad-5/7* strains were derived from an original cross 1358–18d × WT4 2.23c, following three generations of interascus crosses. The *ad-5*, *ad-7* and *ad-5/7* tester strains were kindly supplied by DR. D. WILKIE.

The complete, minimal, and sporulation media used were the same as those given by Cox and BEVAN (1962). The medium used for testing mutant strains for their adenine requirement was minimal supplemented with 0.25 ml of a 0.0074 M solution of adenine per 20 ml medium.

ad-5/7 mutants were isolated as white sectors or whole colonies from platings of red *ad-2.0* cells treated with either UV or EMS. When treated with UV the red cells were suspended in 9 ml of isotonic saline which was then poured into a Petri dish and gently agitated for 3 minutes at 40 cm from a Hanovia Model II Bactericidal lamp emitting 85 percent of its radiation at 2537Å. This dosage gave approximately 10 percent survival.

Treatment of cells with EMS was carried out for 2 hours in a freshly prepared 1 percent solution in saline in a universal screw-top container. Before adding the cells, the solution was shaken vigorously to ensure complete dispersion of the mutagen. During treatment the container was placed in a water bath at 28°C and shaken constantly. Following this, 1 ml was removed from the container and, before plating, was diluted in saline by a factor of at least 10, in order to minimise the effect of any remaining EMS. The pH of the solution fell from 7.0 to 2.8 during this treatment, and approximately 29 percent of the cells survived.

Appropriate dilutions of treated cells were plated on complete medium and incubated for 4 days at 28°C. The resulting colonies were screened for white pigmentation and all suspected mutants subcultured. These were replica plated (LEDERBERG and LEDERBERG 1952) on to minimal medium and minimal plus adenine. This served to distinguish back mutants at the *ad-2* locus (which are white and will grow on both media) from white strains which arise owing to a mutation at a locus prior to *ad-2* in the pathway of adenine synthesis, and which, therefore, still possess the initial adenine requirement of the parental *ad-2.0* strain.

In order to screen the white adenine-requiring strains for *ad-5/7* mutants, as distinct from mutants at other adenine loci, they were replica plated on to plates of minimal medium already spread with a lawn of cells of the opposite mating type of each of the tester strains *ad-5–7*, *ad-5*, and *ad-7*. Those which did not form a prototrophic diploid when crossed with any one or more of these three tester strains, were classified as *ad-5–7*, *ad-5* or *ad-7* mutants according to the growth patterns. Those which did form prototrophic diploids when crossed with all three tester strains were retained for further studies on complementation at the other loci, which act prior to the *ad-2* locus, namely *ad-3*, *ad-4*, and *ad-6*. Before testing the *ad-5/7* mutants for complementation, they were separated from the *ad-2* allele and obtained in both mating types by crossing single cells with wild-type haploids, dissecting asci from the resulting diploid, and selecting the white segregants from the nonparental ditype asci.

The complementation tests were similar to those evolved by BEVAN and WOODS (1963) who reported complementation at the *ad-2* locus. Master plates containing usually 30 mutant strains per plate were prepared. These were replicated on to a series of complete plates on to each of which had been spread a lawn of cells of a different single mutant strain of opposite mating type to those being replicated. These plates were then incubated 18 to 20 hours at 28°C to ensure mating and the formation of diploid cells. At the end of this time the plates were replicated on to minimal medium and incubated for four days before scoring for growth, the presence of which indicated that complementation had taken place. The results of all crosses were recorded in the form of a matrix from which the complementation map was derived.

RESULTS AND DISCUSSION

The isolation of mutants: No spontaneous mutants were isolated from control platings of untreated cells. Of a total of 105 *ad 5/7* mutants isolated, 27 were induced by UV, and 78 by EMS. All were classified into *ad-5*, *ad-7*, or *ad-5–7* mutants on the basis of their complementation reactions with standard strains. The numbers in each category are shown in Table 1 together with the results of ROMAN (1956a, 1956b) and DORFMAN (1963), both of whom recovered spontaneous *ad 5/7* mutants.

Although the number of UV mutants isolated by us is small, it is, we think, sufficiently large to indicate that differences exist in the relative frequencies of the different kinds of mutants produced by UV and EMS. Only 15 percent of the UV are of the *ad-5* type whereas 35 percent of the EMS mutants are of this type. Similarly almost 80 percent of the UV mutants compared with only 58 percent of the EMS mutants are of the *ad-7* type.

The most striking difference between our results and those of ROMAN and DORFMAN is in the relative frequencies of the *ad-5–7* type of mutation: when their results are taken together it is seen that almost 50 percent of their mutants were of this type as compared with approximately 8 percent of ours. By far the majority of our mutants were of the *ad-7* type, which made up 63 percent of our induced mutants compared with 33 percent of their spontaneous mutants.

The difference in relative mutant frequency between our results and those of DORFMAN and ROMAN on the one hand, and between our UV and EMS induced mutants on the other, are worthy of comment. First, in isolating their mutants both ROMAN and DORFMAN employed a technique which favoured the recovery of mutants which could compete successfully with parental red *ad-2* cells during the colony growth when the mutation arose. It will be recalled that their mutants were isolated from white sectors or papillae which arose spontaneously in aging red colonies. Our technique eliminated this selection to a large extent by virtue of the fact that single treated cells were plated. As a result, it turned out that approximately one half of our mutants were isolated from whole white colonies where there was no intra-colony competition of the mutant cells with those of the parental *ad-2* cells. We suggest that this partial elimination of competition between mutant and parental cells may account, at least in part, for the difference between our results and those of ROMAN and DORFMAN. We are unable to confirm this suggestion, however, since all our mutants, both from sectors or

TABLE 1

Distribution of ad-5/7 *mutants derived spontaneously and with UV and EMS*

Locus	Mutagen		Spontaneous	
	UV	EMS	(ROMAN)	(DORFMAN)
ad-5–7	2	6	11	167
ad-5	4	27	8	45
ad-7	21	45	6	107

whole colonies were isolated together and not kept in two distinct groups prior to further classification.

Secondly, and more probably, the difference in the results of the different mutant isolation procedures may be accounted for by the now well established fact that both the sites of spontaneous mutation and those induced by the different kinds of mutagens fall into distinct "hot-spot" distributions when mapped by recombination analysis. Such hot spots were found by BENZER (1961) who compared the distribution of 308 distinct sites produced both spontaneously and by a variety of different chemical mutagens at the *rII* locus of the bacteriophage T4. A similar situation was found by LEUPOLD (1961) and GUTZ (1961) who mapped mutant sites induced by UV, X rays and nitrous acid at the *ad-7* locus of *Schizosaccharomyces pombe*. The mapping of the different *ad-5/7* mutant sites by recombination analysis has not yet been carried out owing to the technical difficulties in obtaining random ascopore suspensions in Saccharomyces. It would seem, however, that at least the differences in the relative frequencies of the *ad-5*, *ad-7*, and *ad-5-7* mutants produced by UV and EMS may be related directly to the mutagens themselves since the same technique was employed for their recovery.

Thirdly, the difference between our induced and ROMAN and DORFMAN'S spontaneous mutant spectra may be due to the fact that spontaneous mutations are qualitatively different from induced mutations. If, for example, spontaneous mutations were disproportionately nonsense or reading frame shift mutations (BRENNER, BARNETT, CRICK and ORGEL 1961), these might more often be *ad-5-7* mutations than would otherwise be expected.

In spite of the lack of recombinational data, differences in the frequencies of different types of *ad-7* mutants produced by UV and EMS are apparent from the patterns of their complementation reactions. This point will be discussed more fully later.

Complementation tests: Of the 105 *ad-5/7* mutants isolated, 97 have been tested for their complementation patterns. Of the remaining eight, five were UV-, and three were EMS-induced. (The former were lost through contamination of stock cultures before they could be tested further; one of the latter was too leaky to distinguish results satisfactorily, and the other two gave very poor mating reactions which, we feel, justified their elimination.) The determination of the complementation patterns was made by testing the mutants in all possible pairwise combinations, even those classified by the standard *ad-5* and *ad-7* tester strains as noncomplementers. In this way it was hoped that anomalous results would not be selected against.

The results of testing the complementation reactions of 7 *ad-5-7*, 31 *ad-5*, and 59 *ad-7* mutants are summarised in Figure 1. We have presented the data for all mutants which gave a qualitatively different complementation pattern. Groups of mutants showing similar patterns are represented by the one which was first shown to exhibit this pattern although there are often quantitative differences in the responses between the members of any group. The number of mutants within each group is noted in parenthesis in Figure 2 which illustrates the complementation map.

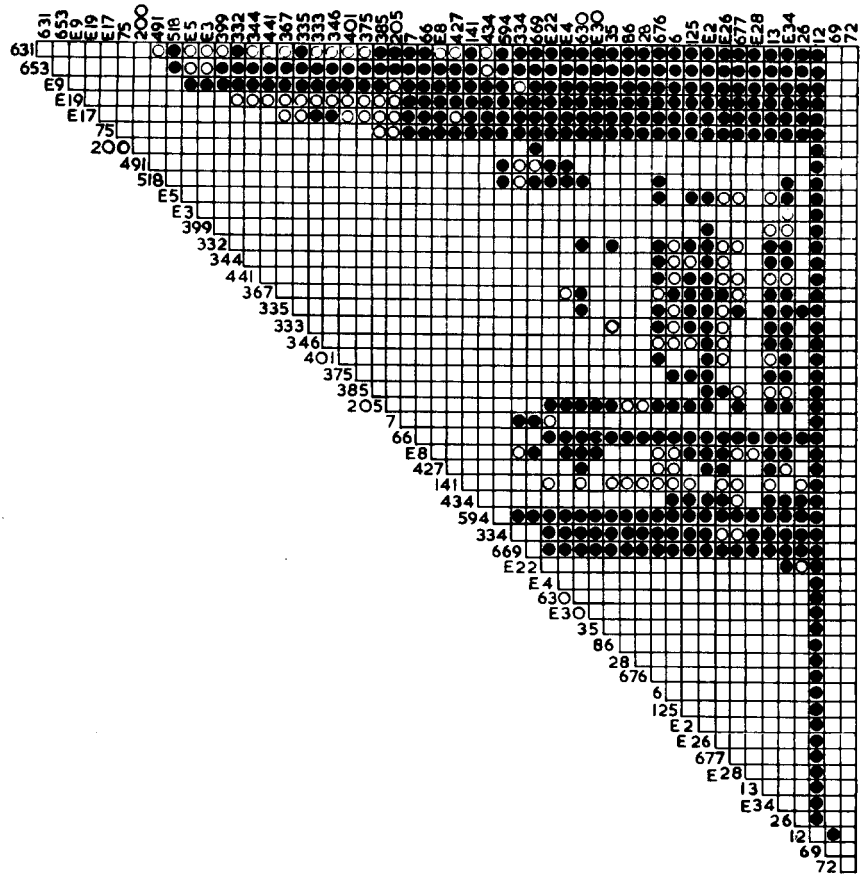

FIGURE 1.—Results of pairwise complementation tests of *ad-5/7* mutants. Good growth ●; poor growth ○; no growth □.

The complementation map shown in Figure 2 is a topographical representation of the results. It is significant in that in its simplest representation it is a circle with a short tail segment, and this differs from all complementation maps previously described. The circle incorporates 22 subunits and the tail segment, 6 subunits of the map. It should be pointed out, however, that when a separate map of UV-induced mutants alone is drawn it is linear with distinct end points, whereas the EMS mutants alone give a map similar to the combined map shown in Figure 2.

Also included in Figures 1 and 2 are the complementation patterns of 15 mutants involved in ten anomalous tests: 333 × 35, 205 × E26, E5 × 6, 375 × E26, E8 × E22, 205 × E28, 141 × 28, 141 × E2, 141 × 12 and 141 × E34. These tests have been repeated and the results are consistent. It should be noticed that of the ten tests, four involve mutant 141, and three involve 205 and E26, each of which appears twice. Bearing in mind that there are no real criteria other than simplicity that state which results should be regarded as correct or as anomalous, we

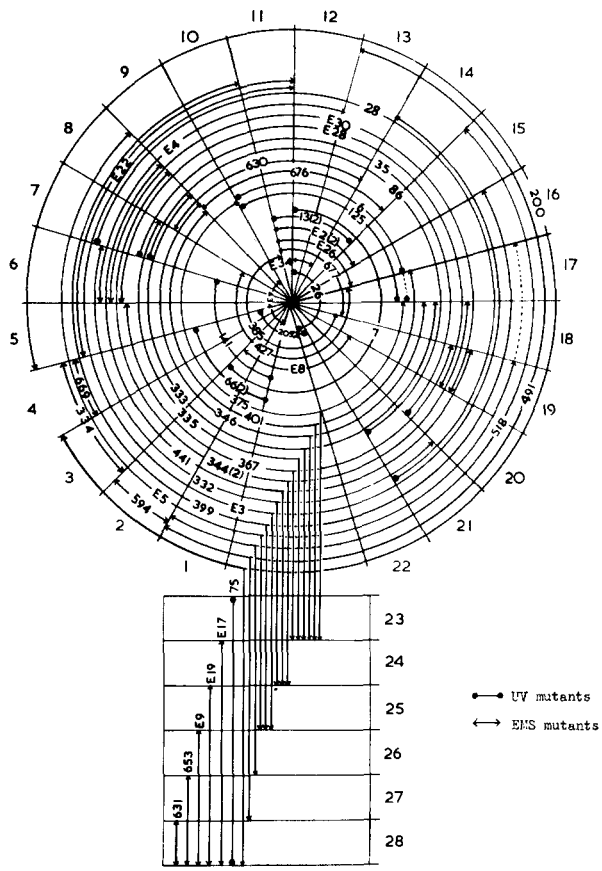

FIGURE 2.—Complementation map of the *ad*-7 mutants.

have placed all 13 mutants in positions on the complementation map which portray the least number of anomalous results. To illustrate this point, consider the position of mutants E5 and 6. In Figure 2 these are shown by dotted lines to overlap one another, which would be consistent with the result of the cross E5 and 6. However, by doing this it can be seen that there now exist three anomalous results, E5 × 677, E5 × 125, and 6 × 427. Consequently the positions of E5 and 6 are shown by continuous lines. Similar situations arise in respect to the placing of the other 13 mutants.

We should like to expand on possible reasons for these anomalous results. Several recent studies have established the corrections of the protein-protein interaction hypothesis for intragenic complementation. In its simplest terms this supposes that complementing mutant alleles produce different variants of the same polypeptide chain and that enzyme activity is given by the mixed aggregate of these chains (FINCHAM 1960). FINCHAM and CODDINGTON (1963) working with complementing mutants of the glutamate dehydrogenase (*am*) locus of *Neurospora crassa* have demonstrated that complementation can occur *in vitro* between

two purified mutant proteins, and that the resulting protein showing enzyme activity contains material from both. Similarly SCHLESINGER and LEVINTHAL (1963) have reported *in vitro* complementation with purified enzyme proteins obtained from alkaline phosphatases. Structural mutants of *E. coli* producing defective varieties of alkaline phosphatases have also been demonstrated by GAREN and GAREN (1963).

It follows from these studies that at least in those complementing mutants studied, the enzyme subunits specified by each allele are bound together to form a protein possessing some enzyme activity. But it does not follow that the cause of noncomplementation between the product of two alleles is the failure of the mixed aggregate to possess enyme activity. In view of the general agreement among the complementation patterns of the vast majority of our *ad-7* mutants, we suggest that such anomalous results as we obtained may be explained on the basis of faulty bonding of enzyme subunits rather than on faulty functioning once bound. This is to some extent borne out by the observation that all but one of our anomalous results are negative, when according to the general map position of the mutants involved, they should be positive. We suggest that in such pairings the respective enzyme subunits cannot hold together to form the polymer although if they could bond they would be functional, as is demonstrated by their functional pairings with other mutants. However, we have no evidence to support this suggestion and in view of the paucity of knowledge on the structure of the enzyme involved such speculation is, perhaps, untimely.

It will be recalled that the mutant strains which we isolated were classified into three groups, *ad-5*, *ad-7*, and *ad-5-7*, on the basis of their complementation reactions with standard tester strains. The complementation reactions of mutants within these groups show a distinct pattern: all *ad-5-7* mutants are complete noncomplementers—they neither complement one another nor any of the mutants of the other two groups, *ad-5* and *ad-7*; *ad-5* mutants complement all *ad-7* mutants, but there is no case of one *ad-5* mutant complementing another *ad-5* mutant; *ad-7* mutants may complement among themselves. In Figure 1 the complementation pattern of *ad-5* mutants is represented by mutant No. 12, and the *ad-5-7* mutants by mutant No. 72. Should all the mutant strains isolated by us be considered to represent different mutant alleles of the same gene locus then a unique situation is encountered. In the complementation map of this locus, we have a specific type of subunit represented by the *ad-5* group of mutants which is overlapped by the noncomplementing group of *ad-5-7* mutants and nothing else. This subunit has not been represented on the complementation map (Figure 2) as there is no justification for assigning it to any particular position on the map.

On the other hand, one may regard each of the *ad-5*, *ad-7*, and *ad-5-7* groups of strains as mutants of three separate genes within an operon (JACOB and MONOD 1961). Should this be the situation then the unique patterns of complementation which have been encountered with the three groups of mutants could be accounted for. *ad-5-7* alleles could be mutations of an operator gene which show no complementation amongst themselves, and which would present the formation

of both *ad-5* and *ad-7* gene products and account for their noncomplementing reaction with all mutant alleles of these genes. *ad-5* mutant alleles could be those of a structural gene which, in common with those of many other loci, show no complementation among themselves, and *ad-7* those of another structural gene some of whose alleles complement one another. Further the frequency of both spontaneous and induced *ad-5-7* mutants relative to the other two groups would become more understandable (Table 1). If only two gene loci, *ad-5* and *ad-7*, were involved then the *ad-5-7* mutants would have to be considered as deletions or as two-gene mutational events. Alternatively, we cannot rule out that these two genes may be read as one from one direction. In this case, a break in transcription along the way could lead to the loss of both gene functions. Although the operon hypothesis is the most attractive one to account for our present data, the knowledge as to whether our strains include mutant alleles of one, two, or three gene loci will have to await further studies on genetic recombination and/or the enzyme(s) involved. As a first step in this direction, we are at present attempting to carry out recombination studies using representatives of each of the three groups of mutants.

A comparison of the complementation patterns of UV and EMS induced ad-7 *mutants:* This has been done by comparing the percentages of the two kinds of mutants which affect each of the subunits on the complementation map (Figure 2). The results are shown in the form of a histogram in Figure 3. It can be seen that the distribution of the mutants induced by each mutagen is not random among the different subunits. To take specific examples, only 6 per cent of the UV induced mutants affect subunit 5 whereas 53 percent affect subunit 12. Similarly 16 percent of the EMS induced mutants affect subunit 26 compared with 53 percent affect subunit 1. Further, it is found that some of the subunits are affected much more by one mutagen than the other. Considering an extreme case, it is found that 53 percent of the EMS mutants affect subunit 6 whereas 6 percent

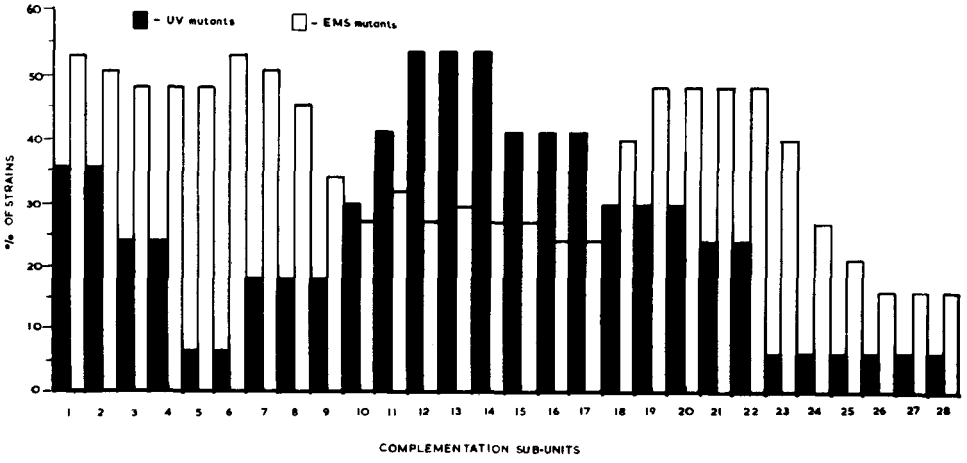

FIGURE 3.—A comparison of the frequencies of UV and EMS induced mutants affecting each complementation sub-unit.

of the UV mutants affect this subunit. Moreover, it can be seen from Figure 3, that taking the map as a whole there is a more general response to the two mutagens embracing a region from subunits 10 to 18 inclusive. That is, within these nine subunits there is an overall decrease in sensitivity to EMS-induced mutations to about half the level of the remaining subunits within the circle. At the same time there is an increase in sensitivity within this region to UV-induced mutations to about twice the average level of the remainder of the map. Thus we may conclude that particular regions as well as specific subunits of the complementation map are affected more by one mutagen than the other. This is reminiscent of the "hot-spot" distribution of mutated sites as determined by genetic recombinational analysis, and it is tempting to make the further conclusion that here we have an additional means of demonstrating that mutated sites induced by different agents are not distributed at random. However, without recombinational data, it is perhaps too early to draw this parallel too far. It does seem significant, however, that for nine adjacent subunits there is a differential response to each of the mutagens, and moreover the responses are reciprocal in nature.

Finally it can be said that, since different complementation subunits are affected more by one mutagen than another, it is clearly advantageous for complementation maps to be constructed from the patterns of a wide spectrum of mutants induced by as many different agents as possible.

One of us (W.P.C.) is indebted to the Department of Scientific and Industrial Research for a Research Studentship, during the tenure of which this work was carried out.

SUMMARY

105 strains mutant at the *ad-5/7* locus have been isolated. Twenty-seven were induced by UV and 78 by EMS. On the basis of their complementation reactions in diploids derived by crossing each of them with (a) standard tester strains, and (b) one another, three types of mutant alleles are distinguished, termed *ad-5*, *ad-7*, and *ad-5-7*. The term *ad-5/7* is used for unclassified mutant alleles. Our induced *ad-5/7* mutations include relatively fewer *ad-5-7* strains than were isolated as spontaneous mutants by ROMAN (1956a) and DORFMAN (1963).

The complementation reactions of seven *ad-5-7*, 31 *ad-5*, and 59 *ad-7* mutant strains were determined. None of the *ad-5-7* strains shows complementation either among themselves or with any of the *ad-5* or *ad-7* strains; they are total non-complementers. All *ad-5* strains behave as a single complementation subunit: they never complement each other, but all of them complement all *ad-7* strains. *ad-7* strains fall into many complementation subunits. In its simplest representation, the complementation map is a circle with a short tail segment; it thus differs from all other maps previously described. The circle incorporates 22, and the tail segment 6 subunits of the map. The UV-induced mutants alone give a linear map, whereas the EMS mutants alone give a map similar to that obtained from the combined results. Fifteen *ad-7* mutant strains gave anomalous results in complementation tests. These were mapped so as to portray the least number of anomalous results. Most of the anomalous results are negative, when according to their general map position they should be positive (i.e. they should complement cer-

tain strains); they may be due to faulty bonding of enzyme subunits rather than faulty functioning of the enzyme once bound. It is suggested that the three types of allele *ad-5*, *ad-7* and *ad-5-7* may be the result of mutations at three different genes constituting an operon, i.e., that *ad-5/7* is not a single locus. The high number of mutant *ad-5-7* mutant strains obtained could then be accounted for by mutation of an operator gene. The comparative complementation patterns of UV and EMS induced mutants suggest that particular regions as well as particular subunits of the complementation map are affected more by one mutagen than the other.

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