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*COMPARATIVE COMPLEMENTATION AND GENETIC MAPS OF THE PAN-2 LOCUS IN NEUROSPORA CRASSA**

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Present evidence in *Neurospora*[1-3] indicates that the phenomenon of complementation between allelic mutants is of widespread occurrence and that this process involves interactions occurring in the cytoplasm between defective gene products, probably polypeptides.[4] Furthermore, it is now clear that the patterns of interallelic complementation at certain loci can be described in terms of linear (one-dimensional) sequences of complementing mutants which have been designated *complementation maps*.[1, 3, 5] This discovery of a non-genetic functional test which establishes a linear sequence of allelic mutants strongly suggests that such a complementation map may reflect the linear organization of both a gene and its product. Such an hypothesis can be tested at loci where both complementation

mapping and genetic (fine-structure) mapping—based on interallelic recombination tests—can be performed, and this paper will describe the results of such a comparative study at the *pan-2* locus in *Neurospora crassa*. A brief, preliminary description of some of these results has been presented previously.[1]

Earlier studies established the occurrence of interallelic complementation as well as genetic recombination at the *pan-2* locus,[6] and subsequent investigations, which will be presented in detail here, led to the development of a complementation map of this locus. Biochemical evidence indicates that this locus in *Neurospora* controls the conversion of keto-valine to keto-pantoic acid.[6] Comparable pantothenic acid-requiring mutants in *Escherichia coli* have been shown to lack activity for a single enzyme.[7] Although similar conclusive evidence has not yet been forthcoming in tests of *Neurospora* mycelial extracts, it seems reasonable to assume that in this organism all *pan-2* mutants also lack or have impaired activity for the same single enzyme. Studies with other *Neurospora* mutants have demonstrated that interallelic complementation occurs at loci known to control the formation of single enzymes.[8-10] Hence, investigations of interallelic complementation appear to have considerable implications for an understanding of mechanisms responsible for the genetic control of protein synthesis.

TABLE 1

ORIGIN AND CLASSIFICATION OF PAN-2 MUTANTS

	Mutagen Used					
	None		X-ray		Ultraviolet	
Strains used	No. of mutants	Number complementing	No. of mutants	Number complementing	No. of mutants	Number complementing
74A	2	1	18	5	15	8
5.5A	2	2	5	2	2	0
3.1a	0	0	11	2	..	..
1167.4A	0	0	3	0	..	..
145.1A	0	0	2	1	..	..
74A-Y230-M138	2	0	13	2	0	0
Total	6	3	52	12	17	8
% complementing	..	50%	..	23%	..	47%

Complementing mutant numbers: (1) Spontaneous origin—from *74A: B3;* from *5.5A: B19, B20;* (2) X-irradiated conidia—from *74A: B5, B7, B28, B36, B39;* from *5.5A: B23, B25;* from *3.1a: B9, B10;* from *145.1A: B43;* from *74A-Y230-M138: B57, B61;* (3) Ultraviolet-treated conidia—from *74A: B65, B69, B70, B71, B72 B73, B74, B75.*

Materials and Methods.—The present studies have utilized a group of 75 pantothenic acid-requiring mutants at the *pan-2* locus (commonly referred to as "pan" B mutants) isolated by the filtration-concentration technique from untreated or from X-irradiated or ultraviolet-treated macroconidia of several different, closely related pantothenic acid-independent strains (Table 1). The standard wild type strain 74A (obtained from Dr. Patricia St. Lawrence) was inbred to produce the two additional wild type strains, 5.5A and 3.1a. The next two strains, 1167.4A and 145.1A, were obtained as pan prototrophs in asci from crosses between two *pan-2* mutants. The first occurred in an ascus in which reciprocal recombination within the *pan-2* locus had occurred, the reciprocal double pan mutant isolate being present, whereas the second isolate (145.1A) was from the ascus in which the reciprocal double pan recombinant was absent.[6] The last strain, 74A-Y230-M138, is an *ad-3B* mutant of spontaneous origin (in strain 74A), in which pan mutants were recovered as double pan-adenine mutants. Only three of the 75 mutants (B1, B34, and B72) are classi-

fiable as leaky on the basis of an ability to grow in the absence of pantothenic acid, and these grow only very slightly.

Initial tests for interallelic complementation employed mixed conidial suspensions on pantothenic acid-free agar plating medium, essentially in the manner described by de Serres.[11] Additional tests for complementation have utilized interallelic crosses of pan mutants, where the presence or absence of pseudo-wild colonies[12] serves to distinguish between complementing and noncomplementing mutant combinations. Tests for pseudo-wilds have proved especially useful in checking the results of conidial tests for the occurrence or non-occurrence of complementation between mutants closely adjacent on the complementation map; i.e., whether such mutants should be considered as affecting two distinct functional regions which are adjacent but do not overlap (complementing types) or as affecting regions which do overlap (non-complementing types). Whereas the results of complementation tests with such mutants utilizing conidia are sometimes variable (whether a given test is positive or negative appears to depend on a number of factors, such as conidial age and concentration), the distinction between the presence or absence of pseudo-wilds in critical crosses is unequivocal and regularly reproducible. Thus, in crosses between closely adjacent mutants which complement, pseudo-wilds regularly grow much more slowly than, and are easily distinguishable from, pan prototrophs arising by genetic recombination. By contrast, in crosses of mutants widely separated on the complementation map, pseudo-wilds are regularly indistinguishable phenotypically (with the exception of those mutant combinations forming temperature-sensitive heterocaryons) from homocaryotic pan prototrophs arising by genetic recombination. Pseudo-wilds can, however, be identified either by further crossing or by conidial plating tests, which serve to establish their heterocaryotic nature.

The methods used to establish the genetic relationships of complementing and non-complementing mutants in terms of a recombination map of the *pan-2* locus have been discussed in detail previously[6] and will not be elaborated here.

Results.—Complementation map of the pan-2 locus: All 75 *pan-2* mutants were tested in all pairwise combinations for their ability to complement. At least one positive response was obtained with 23 of the 75 mutants. The remaining 52 mutants failed to complement in any of the combinations tested and can be placed in a single category designated "non-complementing mutants." The failure of such mutants to exhibit interallelic complementation is not due to the presence of factors preventing heterocaryon formation, since such mutants readily form heterocaryons with other mutants blocked in different biochemical reactions. The relative frequency of complementing to non-complementing mutants appears to be related to the type of mutagen used, since mutants obtained from conidia exposed to X rays yield a smaller percentage of complementing types than do mutants from ultraviolet irradiated conidia (Table 1). Such a relationship may well result from the production by X rays (as contrasted with ultraviolet) of a larger percentage of primary mutants having extensive genetic damage, such as deletions or other associated gross chromosomal rearrangements. In the present sample of *pan-2* mutants, however, there is no clear evidence that mutants arising from X-irradiated conidia are more stable than those derived from ultraviolet-treated conidia.[6, 13]

The 23 mutants which do complement comprise 12 different groups (types)—

with from 1 to 6 mutants in each group—when classified on the basis of the pattern of complementation which they exhibit. The particular pattern of complementation of a given mutant is a genetic property of that mutant, typically exhibiting regular Mendelian segregation in crosses with wild type, or with other *pan-2* mutants.[6] Indeed, extensive crossing tests to wild type of both complementing and non-complementing *pan-2* mutants have produced no evidence that the pattern of complementation characteristic of a given mutant can be modified by recombination, provided a cross is not segregating for factors affecting heterocaryon formation. In crosses between various *pan-2* mutants, however, recombination may occur, as will be discussed later.

The relationships of the 12 different types of complementing *pan-2* mutants can be described in terms of a complementation map of the *pan-2* locus, as shown in the upper half of Figure 1. The construction of such a map depends on the

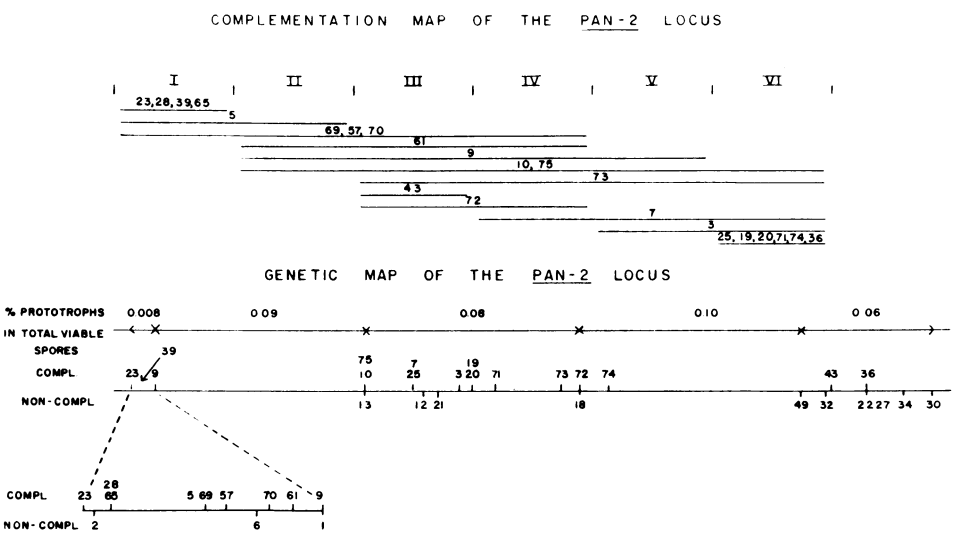

FIG. 1.—Comparative complementation and genetic maps of the *pan-2* locus. Numbers refer to individual *pan-2* mutans. On the genetic map, complementing mutants are indicated above the line, non-complementing ones below. The region between mutants *B23* and *B9* has been enlarged below the main map in order to show the positions of these mutants within this short region.

existence of complementing mutant types which fail to complement with two or more other types.[1, 3, 5] The use of such multiple "overlapping" types permits an ordering of mutants on a map, on the assumption that apparent non-functional regions in such multiple types are regularly continuous, rather than interrupted. In the present instance, the 12 types of *pan-2* mutants can be arranged in a unique linear (one-dimensional) sequence with all of the nine different multiple types represented as continuous. In the currently available group of *pan-2* mutants, 8 of the 12 types are represented by a single mutant only. Clearly, a larger sample of mutants would be highly desirable to confirm the presently indicated order. However, it is presumably significant that any change in the present order would require that certain multiple types be represented as interrupted rather than as continuous. Genetic evidence, to be presented later, supports the view that all of the multiple mutant types are single rather than double mutants.

The initial qualitative tests for complementation of *pan-2* mutants utilized mixed conidia. In general, such tests give reproducible results, and can be scored as either positive or negative. However, in certain combinations, difficulties arise, particularly in those pairs involving multiple mutants which are located in adjacent regions on the map. Such combinations are regularly slow in forming and are often erratic in repeated tests. More definitive tests for complementation between such mutants are provided by crosses where the presence or absence of psuedo-wilds can be detected, as discussed previously. Table 2 indicates the results of crosses made to test both for the expected formation of psuedo-wilds between adjacent, but non-overlapping mutants, and for the expected failure of pseudo-wild type formation between overlapping mutants. The results serve to confirm the initial heterocaryon data and to establish more firmly the boundaries of the functional units on the complementation map.

TABLE 2

EVIDENCE FOR THE PRESENCE OR ABSENCE OF COMPLEMENTATION BETWEEN VARIOUS TYPES OF COMPLEMENTING MUTANTS BASED ON TESTS FOR THE FORMATION OF PSEUDO-WILDS IN CROSSES

Complementing types	I	I-II	I-IV	II-V	II-VI	III	III-IV	IV-VI	V-VI	VI
VI (19, 20, 25, 36, 71, 74	+*	+*	+	+	+	0	+*	+*	0	0	0
V-VI (3)	+*	+	+	+	0	0	+*	+*	0	0	
IV-VI (7)	+*	+	0	0	0	0	+*	0	0		
III-IV (72)	+*	+	#	0	0	0	0	#			
III (43)	+*	+	0	0	0	0	#				
II-IV (10, 75)	+	0	0	0	0	0					
II-V (9)	+	0	0	0	0						
II-IV (61)	+	0	0	0							
I-IV (69, 70, 57)	0	0	0								
I-II (5)	0	0									
I (23, 28, 39, 65)	0										

* Pseudo-wilds occur in crosses. (In combinations marked +*, complementation responses are strong and pseudo-wilds are not phenotypically distinguishable from pan prototrophs; hence routine crossing tests were not made with all these combinations. In other combinations, complementation responses are generally weaker and pseduo-wilds can be distinguished from prototrophs. In most of these instances, crosses were made of all possible mutant combinations.)

0 Pseudo-wilds do not occur in crosses. (Tests within a particular type include both selfings and intragroup crosses.)

\# No cross obtained.

The present complementation map of the *pan-2* locus can be interpreted as indicating the existence of six functionally distinct regions at this locus. However, only three of these regions are defined by single mutant types, the existence of the other three being inferred by the occurrence of "overlaps" of multiple types. The present map thus predicts the existence of three additional single types, which presumably could be obtained in a sufficiently large sample of *pan-2* mutants. Evidence for the existence of analogous single types initially predicted by multiple type overlaps has been obtained at the *ad-4* locus.[5]

A complementation map appears to be an adequate formal representation of the qualitative relationships among complementing mutants. However, it does not directly indicate that there are also significant quantitative differences in complementation exhibited by various pairs of mutants, and that such differences can, in general, be related to the relative positions of any pair of mutants on the map. The majority of *pan-2* heterocaryons formed between mutants separated by one or more regions on the complementation map form within 24 hr after conidial

mixing and grow at 25°C on a medium lacking pantothenic acid at rates (measured as 2-day dry weights or as linear growth rates) comparable to (or in certain instances in excess of) that of wild type. However, by contrast, heterocaryons formed between mutants in adjacent regions on the map (with one exception—mutant *B43* plus mutant *B7*) form more slowly, taking 48 hr or longer before initiation of visible growth on minimal agar plates at 25°C and tend to grow more slowly.

Heterocaryons formed with mutant *B3* or *B7* are somewhat exceptional in that few achieve a wild type growth rate. However, all heterocaryons of these mutants with non-adjacent mutants form at a typically rapid rate (with the exception of the *B7-B5* pair). An additional indication of the unusual character of the *B3* and *B7* mutants is the effect of temperature on the growth rate of heterocaryons involving either of these two mutants. All heterocaryons with *B7* exhibit an unexpected type of temperature-sensitivity in that they grow more slowly at 35°C than at 25°C. Certain heterocaryons involving *B3* (those with *B23*, *B28*, *B39*, and *B65*) exhibit a similar partial temperature-sensitivity, while others (e.g., those with *B5*, *B43*, and *B72*) are completely temperature-sensitive, failing to grow at all at 35°C in the absence of pantothenic acid. The fact that mutants *B3* and *B7* share a common region (region V) on the complementation map may well be a significant factor in the similar unusual properties exhibited by their heterocaryons. Additionally, the fact that both of these mutants are stable (do not revert) and are characterized by aberrant recombination behavior (as will be discussed later) suggests that both may carry extensive genetic damage.

Genetic map of the pan-2 locus: Previous studies of *pan-2* mutants indicated the occurrence of recombination between alleles and led to the development of a linear genetic map of this locus.[6] These studies also clearly established the existence of two major groups of complementing alleles. With the development of a complementation map, it is now possible to compare the two maps and in particular to determine whether the order of mutants is the same on both maps; i.e., whether the complementation and genetic maps are "co-linear."

With this problem in mind, additional extensive studies of recombination between various *pan-2* mutants have been performed. The precise locations of all 75 mutants have not been determined as yet, but crosses involving 34 mutants have located a minimum of 27 different sites within the *pan-2* locus. Both complementing and non-complementing types have been used in crosses, a particular effort having been made to establish the positions of all the complementing mutants for comparison with the complementation map. The non-complementing mutants used for detailed mapping were selected as ones having no obvious irregularities in recombination values with adjacent markers.[6] In general, most crosses have been made between mutants which do not complement with one another; i.e., between one complementing and one non-complementing mutant, or between two non-complementing ones (including crosses of mutants within the same or overlapping functional groups). This procedure was used since crosses of complementing mutants (particularly two mutants widely spaced on the complementation map) yield numerous pseudo-wilds which in general cannot be easily distinguished phenotypically from true recombinants. All crosses were also segregating for closely-linked markers both proximal and distal to the *pan-2* locus, thus enabling mutants

to be ordered by marker behavior, as well as by additivity.[6] The resulting genetic map of the *pan-2* locus is shown in the lower half of Figure 1.

Before a comparison of the two maps is made, certain general features of the genetic map and of particular mutant crosses should be noted. Distances on the genetic map are given as "per cent prototrophs" in the total population of viable ascospores tested and are not directly equivalent to conventional recombination values, which would be obtained by doubling the present values. This correction has not been made since such a procedure assumes the regular occurrence of reciprocal recombination, which has been shown not to occur in certain interallelic *pan-2* crosses.[6]

On the genetic map diagram, complementing mutants have been placed above the line, non-complementing ones below. The region between mutants *B23* and *B9* at the left end of the map has been enlarged below the main map in order to show the positions of these mutants within this short region.

TABLE 3

RESULTS OF VARIOUS MUTANT CROSSES IN WHICH PAN PROTOTROPHS HAVE NOT BEEN OBTAINED

Cross	No. viable ascospores	No. pan prototrophs
20 ylo × 19 tryp*	544,165	0
19 ylo × 20 tryp	368,650	0
10 ylo × 75	1,075,000	0
72 × 18 tryp	391,700	0
22 ylo × 36 tryp	476,560	0
10 ylo × 13 tryp	1,613,500	0
13 ylo × 10 tryp	57,090	0
65 ylo × 28 tryp	429,640	0
65 ylo × 2 tryp	254,880	2 ylo
28 ylo × 2 tryp	105,840	0
10 ylo × 3 tryp	2,478,000	0
13 ylo × 3 tryp	114,920	0
3 ylo × 13 tryp	942,200	0
3 ylo × 75	424,000	0
7 ylo × 25 tryp	90,045	0
25 ylo × 7 tryp	1,088,713	0

* ylo = yellow, tryp = tryptophanless.

The spacing of mutants on the map is based on relative prototroph frequencies. Mutants placed at the same position are ones between which no recombination has yet been detected (Table 3). Extensive data of this kind are from the crosses of mutants *B19* and *B20*. Since these two mutants both arose spontaneously in the same experiment and behave in an identical manner in reverse mutation and complementation tests, as well as in crosses, the possibility of their common origin from a single mutational event cannot be excluded. Two additional mutants, *B10* and *B25*, which also show identical behavior in complementation tests, differ widely in origin and cannot be the result of a single mutational event. Three crosses (*B72* with *B18*, *B22* with *B36*, and *B10* with *B13*) are of particular interest, since they represent pairs in which one member is a complementing and the other a non-complementing type. In at least one of these instances, however, it is clear that the pairs of mutants differ in other respects, since *B22* is a stable mutant, while *B36* reverts. Mutants *B65* and *B28* apparently constitute another pair exhibiting no

recombination. On the basis of present data, *B28* also fails to recombine with *B2*, whereas the cross of *B65* with *B2* has yielded two recombinants. These results could be interpreted as indicating that mutant *B28* is a deletion of the type utilized in the mapping of the *r*II mutants in bacteriophage T2 by Benzer.[14] However, the number of ascospores tested in these crosses is still too small to warrant such a conclusion. In addition the behavior of mutant *B28* in crosses with *B65* and *B2* is aberrant and strongly suggests that this mutant may be associated with some type of rearrangement. In crosses of *B28* with closely adjacent mutants, no parental type prototrophs with respect to adjacent markers are recovered, whereas such types regularly occur in crosses with mutants at a distance from *B28*. Probably significant also, is the fact that *B28* is a stable mutant.

Certain crosses with two other mutants (*B3* and *B7*) have also yielded no prototrophs. However, in both these instances the available data from other crosses especially with presumptive adjacent mutants, indicate aberrant behavior of these two mutants, implying that they are genetically more complex than localized single site mutants. The proper location of *B3* is particulary difficult to establish. Despite the fact that *B3* fails to yield prototrophs in crosses with

TABLE 4

GENETIC LOCALIZATION OF PAN MUTANTS ON THE BASIS OF CROSSES TO MARKED STOCKS OF B5 AND B3

		Complementing Mutants (grouped as to origin)					Non-Complementing Mutants (grouped as to origin)				
Classification	Total	Spontaneous	X-ray	UV	Total	% Total	Spontaneous	X-ray	UV	Total	% Total
Mutants near *B5*	14	..	7	3	10	71.4%	..	4	..	4	28.6%
Mutants near *B3* or distal to *B3*	59	3	5	5	13	22.0%	3	34	9	46	78.0%
Not classified	2	..	..	..	0	....	..	2	..	2	....
Total	75	3	12	8	23	....	3	40	9	52	....

B10, *B13*, and *B75*, an examination of the frequencies and distribution of prototrophs in numerous other crosses involving *B3*, appears to place it adjacent to *B19* and *B20*. Although *B3* is placed to the left of *B19* and *B20* on the map, present data do not exclude its location to the right. As mentioned earlier, both *B3* and *B7* are stable mutants and are characterized by forming temperature-sensitive heterocaryons with other mutants.

The only complementing mutant not placed on the present genetic map is *B39*. Crosses with *B39* are characterized by low viability of ascospores, but presently available data indicate that this mutant is located at some position between *B23* and *B9*.

In addition to the crosses designated to localize particular mutants as precisely as possible, further crosses were made with all remaining *pan-2* mutants to marked stocks of both *B5* and *B3*. These crosses make it possible to assign mutants to either the left end of the map near *B5*, or to the right half (adjacent to or distal to *B3*). The results (Table 4), taken together with the earlier data, suggest a rather marked asymmetry of the genetic map of the *pan-2* locus in two respects. A disproportionately high number of mutants is concentrated in a relatively short section at the left end of the map. This group of mutants is separated by a rela-

tively long region—in which no mutants have as yet been located—from the remaining mutants, which are rather generally distributed over the right two thirds of the map (with the exception of the space between *B74* and *B49*, in which some of the mutants not yet precisely localized are apparently situated). In addition to this asymmetry in over-all mutant distribution, there is also a marked asymmetry in the distribution of complementing versus non-complementing mutants. Over 70 per cent of the mutants near the left end of the map are complementing types, whereas only about 20 per cent of those to the right on the map exhibit interallelic complementation. Nor does this asymmetry appear to be a result of a difference in mutagenic origin of the two groups of mutants, since the same general type of asymmetry characterizes mutants from ultraviolet irradiated as well as from X-irradiated conidia.

Comparison of the complementation and genetic maps: With the establishment of detailed complementation and genetic maps of the *pan-2* locus, a comparison of the two maps can now be made to determine whether they are co-linear. At first glance there appears to be, in general, a rather striking agreement between the ordering of mutants on the two maps. All mutants (with the exception of *B43*) located exclusively or primarily in regions I through III (the left half) of the complementation map, are also located near the left end of the genetic map. Furthermore, the order of mutants in this region of the genetic map agrees with the order on the complementation map, which is taken to be as follows (from left to right) for the various complementing types: I (*B23*, *B28*, *B39*, *B65*); I-II (*B5*); I-II-III-IV (*B69*, *B57*, *B70*); II-III-IV (*B61*); II-III-IV-V (*B9*). All mutants located exclusively or primarily in regions IV through VI (the right half) of the complementation map are also located in the right portion of the genetic map. There is, however, a marked asymmetry between the two maps, since all mutants in regions I through III(the left 50%) of the complementation map (again with the exception of *B43*) are confined to a region whose length is less than 2.5 per cent of the genetic map, whereas mutants in the right 50 per cent of the complementation map are located in a region which comprises approximately 70 per cent of the genetic map. No mutants, either complementing or non-complementing, have as yet been located in the space between these two regions on the genetic map. These two major groupings of complementing mutants correspond to the "*B5*" and "*B3*" groups detected in the first studies of complementation at the *pan-2* locus.[6]

Although there is a striking general correlation between the ordering of many of the mutants on the two maps, significant exceptions occur, indicating that strict co-linearity does not hold. The most significant mutants for a comparison of the two maps are those that occupy single or double regions on the complementation map, since long multiple types can only be approximately located in terms of the general distribution on the map of the several regions which they occupy. Thus the most significant mutants become those in regions I, III (*B43*), and VI, together with *B72* (III-IV). On the basis of co-linearity, mutants *B43* and *B72* should be located on the genetic map between mutants of complementation Types I and VI. However, initial recombination data from crosses of various single mutants with *B43* and *B72* located these two mutants at quite different positions among single mutants of Type VI. In order to check the positions of these two critical mutants as completely as possible, they were also crossed to three different available double

pan-2 mutants obtained by recombination within the *pan-2* locus[6] (and unpublished). The results of crosses with these double mutants, as well as with the nearest non-complementing single mutants are given in Table 5. Mutant *B72* has been somewhat difficult to work with genetically since it is leaky. In crosses, parental type ascospores of this mutant germinate rather well in the absence of pantothenic acid. However, pan prototrophs obtained in such crosses are distinguishable from the *B72* parent. The presence of pseudo-wilds in crosses of *B72* with adjacent complementing mutants was detected by using a *B72 tryp-1* parent and plating on minimal medium. By means of this procedure, only pseudo-wilds and tryptophane-independent pan prototrophs are recovered on plates.

The results of various crosses of *B43* and *B72* with other single mutants—with

TABLE 5

GENETIC LOCALIZATION OF MUTANTS B72 AND B43

Cross	No. viable ascospores	No. pan proto-trophs	Frequency of pan prototrophs	Distribution of Pan Prototrophs*				Indicated order (from marker distribution)
				Non-crossover		Crossover		
				P1	P2	++	ylo tryp	
Single × single								
72 × 18 *t*	379,800	0	0	0	0	0	0	...
12 *y* × 72 *t*	408,000	151	0.04	10	16	66	4	12–72
3 *y* × 72 *t*	58,625	6	0.01	3	3	0	0	...
22 *y* × 72 *t*	294,000	250	0.08	12	15	15	58	72–22
43 *y* × 72 *t*	579,620	475	0.08	26	10	13	51	72–43
Double × single								
5–3 *y* × 72 *t*	111,180	11	0.01	1	0	5	5	...
5–36 *y* × 72 *t*	77,180	3	0.004	0	0	2	1	...
23–36 *y* × 72 *t*	119,340	8	0.006	1	6	1	0	...
Single × single								
3 *y* × 43 *t*	105,750	301	0.28	20	0	80	0	3–43
43 *y* × 18 *t*	399,570	789	0.19	20	20	6	51	18–43
43 *y* × 49 *t*	521,220	54	0.01	7	2	5	40	49–43
32 *y* × 43 *t*	905,760	21	0.002	6	0	15	0	32–43
43 *y* × 32 *t*	379,050	11	0.003	3	2	0	6	32–43
43 *y* × 22 *t*	388,160	54	0.01	4	4	43	3	43–22
43 *y* × 27 *t*	72,420	15	0.02	4	5	5	1	43–27
43 *y* × 34 *t*	155,150	409	0.26	22	26	33	15	43–34
43 *y* × 30 *t*	203,660	320	0.15	30	6	46	18	43–30
Double × single								
5–3 *y* × 43 *t*	517,640	808	0.15	40	0	148	0	5-3-43
5–36 *y* × 43 *t*	683,120	24	0.003	8	0	16	0	...
5–36 *t* × 43 *y*	203,610	6	0.002	0	1	3	2	...
23–36 *y* × 43 *t*	679,320	16	0.003	1	3	11	1	...

y = yellow, *t* = tryptophanless.
* All prototrophs tested if less than 100 recovered; otherwise data are based on tests of a sample of 100.

respect to both prototroph frequencies and marker distribution in the two crossover categories—are generally consistent in placing these mutants as indicated on the genetic map. In addition, the results of crosses with the double mutants support the order indicated. The most significant data are those obtained in the crosses with *B43*. The *B5–B3* × *B43* cross clearly places *B43* to the right of *B3* on the basis of both prototroph frequencies and marker distribution. The *B5–B36* and *B23–B36* crosses with *B43* are also consistent on the basis of low prototroph frequencies in locating *B43* between the two members of these double mutants. Marker distribution, taken as observed, places *B43* to the right of *B36*. However, this effect is very probably related to the pecularities of recombination in such a three point intralocus cross where both conversion and negative interference

are apparently operating,[6] and the effect is probably accentuated by the close proximity of *B43* to *B36*. The quantitative results of double mutant crosses with *B72* are less conclusive, primarily because of the abnormally low recombination values characteristic of all crosses between *B3* and adjacent mutants. However, the prototroph frequency data from the cross of *B5–B3* with *B72*, compared with those from the crosses of *B5–B36* and *B23–B36* with *B72* are consistent with a localization of *B72* to the right of *B3* and to the left of *B36*.

Complementation tests utilizing double mutants: The availability of double mutants within the *pan-2* locus makes possible further significant complementation tests. The responses of various single mutant combinations permit a quantitative prediction as to the responses of various double-single mutant combinations. On the simplest hypothesis, a particular double mutant is expected to complement

TABLE 6

RESULTS OF COMPLEMENTATION TESTS UTILIZING PAN DOUBLE MUTANTS*

Combinations Tested (Singles with singles)	Type of Complementation Test: Conidial mixing	PWTS	Combination Tested (Singles with doubles)	Type of Complementation Test: Conidial mixing	PWTS
43 + 5	++	++	43 + 5–3	0	+
43 + 3	+++	–	43 + 5–36	0	++
43 + 23	+++	–	43 + 23–36	0	++
43 + 36	+++	–	.....	..	..
72 + 5	++	++	72 + 5–3	0	0†
72 + 3	+++	–	72 + 5–36	0	++
72 + 23	+++	–	72 + 23–36	++	++
72 + 36	+++	–	.....	..	..
61 + 5	0	0	61 + 5–3	0	0
61 + 3	++	++	61 + 5–36	0	0
61 + 23	++	++	61 + 23–36	0	0†
61 + 36	++	++	.....	..	..
9 + 5	0	0	9 + 5–3	0	0
9 + 3	++	++	9 + 5–36	0	0
9 + 23	++	++	9 + 23–26	0	0†
9 + 36	++	++	.....	..	..

* Comparisons have been made of various pairs of single mutants with various single-double mutant pairs. Evidence for complementation is based on tests for heterocaryon formation both from conidial mixing and from crosses to detect pseudo-wilds, PWTS.

Symbols used: – Complementation by heterocaryon formation following conidial mixing normal; no routine check made for PWTS. 0 No complementation detected. + Very light response; no further growth on transfer. ++ Intermediate response; further growth after transfer, but wild-type rate never attained. +++ Good response; continuing growth after transfer; rate comparable to wild type.

† Combination expected to yield PWTS on basis of absence of overlapping regions in single-double combinations.

with all single mutants with which it fails to overlap. Actually, four single mutants —*B9*, *B43*, *B72*, and *B61*—which occupy regions in the middle of the complementation map are expected to complement with one or more of the three available double mutants. Hence, tests for complementation with the double mutants were performed with all four of these single mutants, utilizing both conidial mixing tests and crosses to detect the presence of pseudo-wilds. As has already been indicated, this last test is the more sensitive and reproducible of the two tests, especially in instances where the degree of complementation is restricted.

The results of these tests, including all possible pairwise single mutant combinations as controls, are presented in Table 6. It is evident that in the double-single combinations, many more of the anticipated positive responses are obtained in the tests for pseudo-wilds than in conidial mixing tests. In fact, only

one of the conidial mixing tests involving a double gave positive results (*B23–B36* with *B72*). In several cases, unrelated biochemical "forcing" markers were employed to determine if other negative combinations would respond in conidial mixing tests. Although such heterocaryons formed on pantothenic acid-supplemented medium, the resulting heterocaryons would not grow in the absence of this supplement. These results may well be due to an inability of such heterocaryons to attain favorable nuclear ratios permitting a positive complementation response. It is perhaps not surprising that the initiation of complementing heterocaryons is most successful from pseudo-wilds where presumably two homologous chromosomes are present within the disomic nuclei of a single ascospore, a situation which leads to the formation of a heterocaryon having, at least initially, equal numbers of two types of mutant nuclei. In all instances where double-single mutant combinations gave positive responses permitting continued growth after transfer, (i.e., in all combina-

TABLE 7

RESULTS OF CIS-TRANS TESTS WITH VARIOUS PAN-2 MUTANTS*

Heterocaryons	mm/hr	Dry weight in mgs.
Controls		
74A (wild type)	3.2	35.0
B3 + *F4*	3.1	39.9
B5 + *F4*	3.1	42.0
B23 + *F4*	3.3	46.4
B36 + *F4*	3.1	41.6
Trans		
B5 + *B3*	3.0	35.2
B23 + *B3*	3.1	43.7
B5 + *B36*	3.2	45.0
B23 + *B36*	3.1	41.2
Cis		
B5-B3 + *F4* (5)†	3.1	33.5‡
B5-B36 + *F4* (2)	3.2	41.2
B5-B36 + *F4* (11)	3.2	35.9‡
B23-B36 + *F4* (7)	3.1	31.7‡

* Growth responses are given in terms of both 27-hr dry weight and linear growth rates in the absence of pantothenic acid. An *ad-4* mutant (F4) has been used as a forcing marker for the *cis* (pan double mutant combinations) hence control heterocaryons include all *pan-2-F4* pair combinations.

† Numbers in parentheses refer to particular isolates.

‡ Heterocaryon incompatibility factors are probably still influencing these growth responses.

tions except *B5–B3* with *B43*), the resulting heterocaryons were resolved and the two parental types recovered.

In three instances, double-single combinations which were expected to be positive on the basis of the absence of overlapping regions in common yielded no pseudo-wilds (*B5–B3* with *B72*; *B23–B36* with *B61*; *B23–B36* with *B9*). Moreover, microscopic examinations of plates from these crosses indicated no abortive colony formation of the type observed in crosses of *B5–B3* with *B43*. These results are perhaps not entirely unexpected in that in all these instances one or both of the pairwise combinations between a particular single mutant and the members of the double exhibit a much reduced complementation response. In addition, it is clear that even in instances where positive responses are obtained in double-single combinations, these are regularly weaker than would be anticipated from the responses of corresponding single mutant pairs. For example, both *B43* and *B72* grow at wild type rates in heterocaryons with *B23* and *B36*, yet the two corresponding double mutant combinations (*B32–B36* with *B43* and with *B72*) grow at markedly

reduced rates which are considerably less than 10 per cent of the wild type rate. Hence it is evident that when two *pan-2* mutations are simultaneously present in a given strain, they regularly interact to restrict the ability of the strain to complement to the degree expected. This interaction may result either in a weaker complementation response or in the complete absence of detectable complementation.

The availability of double mutants makes possible one further type of comparative complementation test of significance in defining the functional inter-relationships of *pan-2* mutants, i.e., the *cis-trans* test employed by Benzer[14] in defining a cistron. In this test, growth (measured as 27-hr dry weight or as linear growth rate) in the absence of pantothenic acid has been used as the measure of function. In making the *cis* comparison (double pan mutant against wild type at the *pan-2* locus) an *ad-4* mutant (*74A-Y112-M160=F4*) has been utilized as a forcing marker. Consequently an additional set of control heterocaryons has been made between this mutant and the single pan mutants tested. The results of these tests are given in Table 7. The data indicate that all the *trans* combinations tested are equal (or superior) in growth to comparable *cis* combinations, thus providing evidence for functional independence of such single mutants. The reduced dry weights characteristic of certain *cis* combinations (e.g., *B5–B36* and *B23–B36*) probably indicate the presence of genetic factors restricting heterocaryon compatibility (introduced during crossing) rather than an inherent superiority of certain *trans* combinations.

Discussion.—The mechanism of interallelic complementation: The characteristics of complementation between alleles at the *pan-2* locus are fully compatible with the hypothesis that this process involves interactions occurring in the cytoplasm of the heterocaryon between two types of differentially defective gene products derived from two types of differentially defective *pan-2* genes. Although present evidence from *Neurospora* has not yet conclusively established that the *pan-2* locus controls the synthesis of a single enzyme, such an assumption appears reasonable. Since recent *in vitro* studies with mutants at other loci known to control single enzymes make it highly probable that interallelic complementation occurs at the polypeptide level,[4, 15] the further assumption can be made that a similar situation exists at the *pan-2* locus. The actual mechanism of complementation is, however, not yet clear, but could include one or more of the following types of interactions between polypeptides: (1) Dissociation and recombination involving two or more distinct, differentially defective, polypeptide chains of a single protein, as is known to occur in the case of hemoglobin.[16] (2) The union of two different incomplete individually non-functional parts of a single polypeptide chain resulting in the restoration of enzymatic function, as has been shown to occur in the case of ribonuclease.[17] (3) Some type of cooperative interaction involving two differentially defective, polypeptide chains in an enzyme polymer molecule, as suggested by Brenner (unpublished).

The first of these mechanisms has been used in an attempt to explain complementation between mutants at the *ad-4* locus lacking activity for the enzyme adenylosuccinase,[4, 5] particularly on the basis of the maximum level of enzyme found in heterocaryons. However, the apparent presence of numerous distinct functional units at the *ad-4* locus, as at the *pan-2* locus, introduces difficulties for a simple dissociation-recombination hypothesis. Additionally, the simplest form of this hypothesis would apparently require that the enzyme present in complementing

heterocaryons be identical with that in wild type. Recent evidence indicates, however, that the adenylosuccinase present in at least some complementing heterocaryons (those which are temperature-sensitive in their growth response) is quite different from that in wild type.[18] By analogy, a similar situation may well exist in the temperature-sensitive heterocaryons involving *pan-2* mutants.

The second mechanism appears to be incompatible with present evidence from the *pan-2* locus concerning the distribution of complementing mutants on both the complementation and genetic maps. As yet no conclusion can be reached concerning the possible role of enzyme polymers in complementation, since this model has not yet been formulated in precise terms, nor has the structure been determined for any enzyme molecule known to be involved in complementation.

The genetic map of the *pan-2* locus provides significant information for interpreting the large class of "non-complementing" mutants. Formally such mutants could be represented as continuous across all six regions of the complementation map, and might be interpreted as large deletions of the entire *pan-2* locus. Such is clearly not a proper interpretation, however, since many non-complementing *pan-2* mutants are capable of reverse mutation,[6] as is true of similar mutants at other loci.[1, 3, 19] Additionally, these mutants occur at numerous different localized mutational sites throughout the *pan-2* locus, although in the present sample a larger percentage is concentrated near the right end of the map. Perhaps the simplest present interpretation of such mutants is that they make no protein related to the normal enzyme protein controlled by a particular locus, i.e., the mutational change results in the presence of nonsense DNA at the locus.[20] Thus they may be analogous to non-CRM-forming mutants at the loci in *Neurospora* and *E. coli*.[15] Alternatively, such mutants may make a protein which has been changed to such an extent that it is completely incapable of interacting, even with other differentially defective related proteins. The possibility that at least some non-complementing *pan-2* mutants make some enzyme-related protein is supported by the evidence that at least one such mutant (*B1*) is slightly leaky.

The genetic map of the *pan-2* locus also provides some evidence as to the nature of long multiply-complementing mutant types. Again, most of these cannot represent deletions, since most revert, nor are they multiple site mutants on the basis of their localization on the genetic map. An explanation for the "spreading effect" of such mutants is not obvious. One possibility may be that the mutational changes characteristic of such mutants result in mutant enzyme molecules which are incapable of interacting with long, continuous neighboring regions of homologous monomer units in an enzyme polymer.

The genetic map of the *pan-2* locus also indicates that mutants of the same complementation type need not be located at the same site. In fact such mutants may be very widely separated on the genetic map; e.g., mutants *B25* and *B36*. Thus the ability of two mutants to exhibit genetic recombination clearly does not make them capable of exhibiting cytoplasmic interactions resulting in complementation, at least to such a degree that detectable growth in the absence of pantothenic acid can occur. There is also evidence that certain pairs of mutants which are relatively closely adjacent on the genetic map may exhibit a pronounced ability to complement; e.g., *B43* and *B36* or *B72* and *B74*. To date, no pair of mutants at the same site which can complement one another has been obtained, although

three apparently non-recombining pairs exist which include one complementing and one non-complementing member. Additional genetic evidence will be required to determine whether such pairs are indeed incapable of being separated by recombination.

The problem of co-linearity of the complementation and genetic maps: In most current hypotheses of gene action,[21] the base sequence of a particular segment of DNA is assumed to serve as the code (presumably by way of an intermediate RNA template) for a corresponding sequence of amino acids in a polypeptide chain, which then undergoes folding to give the tertiary structure of the final functional protein molecule. On this view, the order and relative positions of mutations on a genetic map should correspond to the order and relative positions of corresponding altered amino acids along the polypeptide chain; i.e., the two sequences should be "co-linear."

If the process of interallelic complementation involves interactions between extended polypeptide chains, then co-linearity of the genetic and complementation maps might be anticipated if on the enzyme polymer hypothesis altered amino acids located in different parts of two otherwise identical unaltered chains (each derived from one of the two different mutant nuclei in a complementing heterocaryon) could interact to produce a functional product. An alternative possibility would be that complementation involves interactions between altered polypeptide molecules which are completely or partially folded. In such a situation, exceptions to co-linearity might be anticipated, since complementation would involve interactions between three-dimensional rather than one-dimensional structures. The exceptions to co-linearity between the two maps of the *pan-2* locus may result from a situation of this kind. In the present instance, the marked co-linearity between the left ends of the two maps is quite striking and could reflect an absence of pronounced folding in one of the terminal regions of this particular enzyme protein. However, an alternative possibility might be that complementation can occur only between mutants in this region which produce altered polypeptides characterized by an absence of normal tertiary structure in the region.

Complications in making comparisons of complementation and genetic maps could arise if certain mutants are associated with chromosomal rearrangements. Although there is no evidence for such an association in the case of mutants *B43* and *B72*, it should be recalled that *B43* is of exceptional origin in being derived from X-irradiated conidia of a pan prototroph (strain 145.1A) which itself arose by "gene conversion." It is evident that additional *pan-2* mutants (preferable of spontaneous origin) localized in single regions of the complementation map would be most useful in further tests of co-linearity between the genetic and complementation maps of this locus.

The apparent one-dimensional character of complementation maps may well be subject to exceptions, although present evidence from the *pan-2* and other loci[3, 5] suggests that such exceptions are infrequent. Clearly, a very large number of complementing allelic mutants is needed to define adequately the character of a given complementation map, particularly in those instances where the presence of several functional regions is indicated.

Functional units at the pan-2 locus: The discovery that interallelic complementation is of widespread occurrence, together with the evidence that this phenomenon

can often be described in terms of complementation maps consisting of several distinct regions, raises major problems in defining a genetic unit in terms of function.

The operational test for complementation used in *Neurospora* is the same *cis-trans* test employed by Benzer[14] in defining the "cistron" as a unit of function. Indeed, in the present studies at the *pan-2* locus, the complete test can be performed with certain mutant combinations, since mutants can be compared in the *cis* as well as in the *trans* arrangement. Such comparisons indicate complete functional independence of various pairs of mutants on the basis of the ability of such pairs in the *trans* arrangement to grow in the absence of pantothenic acid at rates equivalent to their rates of growth in the *cis* configuration. On the basis of such evidence, mutants in at least three regions (I, III, and VI) of the complementation map may be considered to represent three distinct functional groups, as defined by the *cis-trans* test. These groups should correspond to three groups of mutants in three limited continuous segments, or "cistrons," of the genetic map of the *pan-2* locus. However, this expectation is not met, since, although the mutants in region I of the complementation map occur together in one region of the genetic map, mutant *B43* (in region III) is located on the genetic map between mutants grouped in region VI of the complementation map.

Further evidence of unexpected complexities of applying the *cis-trans* test is provided by the existence of multiple (overlapping) complementing types, together with instances of partial rather than complete complementation. Perhaps most significant of all is the evidence that at the *pan-2* locus, as well as at several other loci where complementation occurs, the majority of mutants fail to exhibit any interallelic complementation. Such mutants do, however, regularly complement with other groups of mutants known to be blocked in different reactions and to be located at other loci. The existence of such non-complementing alleles located at numerous different mutational sites within a locus may well prove to be the most significant single indication derived from non-biochemical studies of the fundamental functional unity of a given group of mutants.

Tests for different functional groups of *pan-2* mutants have employed growth responses only, since a satisfactory enzymatic assay for the reaction blocked in these mutants is not yet available in *Neurospora*. Enzymatic tests can, however, be performed at the *ad-4* locus, where previous studies have developed a complementation map similar to that at the *pan-2* locus. In this instance, individual *ad-4* alleles are known to lack activity for a single enzyme—adenylosuccinase. As at the *pan-2* locus, various pairs of mutants can complement in the *trans* configuration and grow in the absence of adenine at rates equivalent to wild type. Such results provide evidence for several groups of functionally distinguishable mutants, which have been tentatively referred to as representing several "cistrons" at the *ad-4* locus.[4, 5] However, complexities similar to those among *pan-2* alleles occur in the complementation relationships at the *ad-4* locus, including the occurrence of non-complementing types. In addition, enzyme assay data have shown that complementing heterocaryons (*trans* configuration) have no more than half the adenylosuccinase activity expected of *cis* heterocaryons (on the assumption that such *cis* heterocaryons should have activities similar to those found in *ad-4*/*ad-4*$^+$ heterocaryons), despite the fact that *trans* heterocaryons grow at rates equivalent to wild type.

Thus, although the *cis-trans* test based on growth responses provides evidence of functional independence of certain groups of allelic *ad-4* mutants, biochemical evidence (based on quantitative assays and qualitative behavior of adenylosuccinase in complementing heterocaryons) indicates functional relatedness.

Clearly, present results of interallelic complementation studies in *Neurospora* demonstrate that considerable complexities exist. Hence attempts to classify functional groupings of related (isolocal) mutants in terms of "cistrons" appear premature. In fact the over-all evidence appears to indicate that the application of the *cis-trans* test in defining a "cistron" requires certain qualifications and restrictions. In his studies of *r*II mutants in bacteriophage, Benzer[14, 22] employed almost exclusively non-leaky, stable mutants, many of which behave genetically as deletions. On the basis of these mutants, evidence has been obtained by the *cis-trans* test for only two groups of functionally independent types, corresponding genetically to the A and B cistrons. However, the use of such a specifically selected group of *r*II mutants, although clearly desirable for recombination tests, may well place serious restrictions on the detection of functional interactions by means of the *cis-trans* test. Such *r*II mutants could well include only types of A and B mutants which are entirely (or largely) incapable of exhibiting intragroup functional interactions, and could thus correspond to *pan-2* alleles occurring in the non-complementing category. On this view, the *pan-2* locus, as well as the *ad-4* locus, would correspond to single "cistrons," as this term is employed in describing the A and B groups of mutants in the *r*II region. The occurrence in T4 of two adjacent genetic regions containing two functionally related, but distinguishable, groups of mutants would thus be more comparable in *Neurospora* to the situation in the *ad-3* region in linkage group I. This region is composed of two functionally quite distinct groups of mutants (*ad-3A* and *ad-3B*) corresponding to two adjacent chromosomal segments.[10] Complementation between members of the two different groups is the general rule, whereas complementation between members of the same group, although relatively rare, does occur (de Serres, unpublished). An alternative interpretation of the *r*II results would be that interactions between intracistron mutants leading to partial or complete restoration of function cannot occur regardless of the type of mutational changes involved, because of the chemical nature of the products of these segments. The existence of loci in *Neurospora* at which interallelic complementation does not occur is still a distinct possibility.[3]

If, indeed, genetic loci in *Neurospora* such as the *pan-2* and *ad-4* are to be considered unitary in some basic functional sense, such as in their control over the synthesis of a particular enzyme protein, and hence are to be considered equivalent to single cistrons, then the results of interallelic complementation studies indicate that the *cis-trans* test can still be employed in the analysis of intracistron mutant relationships. A proper interpretation of the nature and significance of the functional subunits which such studies have demonstrated must await further evidence.

Summary.- The present study has utilized 75 mutants at the *pan-2* locus in *Neurospora crassa*. This locus controls the conversion, in the biosynthesis of pantothenic acid, of keto-valine to keto-pantoic acid, a reaction presumably catalyzed by a single enzyme in this organism. All 75 mutants were tested in all possible pair-wise combinations for their ability to complement in heterocaryons. Twenty-three of the mutants gave at least one positive response, the remaining 52 failing to

complement in any combination. The 23 complementing mutants comprise 12 different groups (type) and these 12 types can be arranged in a linear (one-dimensional) sequence to form a complementation map of the *pan-2* locus.

The relationships of mutants on this complementation map have been compared with their relationships on a genetic map of the locus derived from recombinational analyses. Although there is, in general, a rather marked agreement in the ordering of mutants on the two maps, certain marked exceptions to complete "co-linearity" occur.

Various possible interpretations of interallelic complementation and of the relationship of this phenomenon to genetic fine structure are discussed. Particular consideration is given to the characteristics of interallelic complementation as they relate to the problem of defining a genetic unit of function.

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