GENETIC CONTROL OF ALKALINE PHOSPHATASE SYNTHESIS IN NEUROSPORA: THE USE OF PARTIAL DIPLOIDS IN DOMINANCE STUDIES[1]

ROBERT L. METZENBERG[2], MARY K. GLEASON AND BARBARA S. LITTLEWOOD

Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Manuscript received December 12, 1973

ABSTRACT

In wild-type *Neurospora*, alkaline phosphatase is made under conditions of phosphate limitation, but not conditions of phosphate sufficiency. Mutants at two unlinked loci, *nuc-1* and *nuc-2*, do not make alkaline phosphatase under any conditions, while mutants at two quite closely linked loci, *pcon* and *preg*, make alkaline phosphatase even under conditions of phosphate sufficiency. *pcon* is extremely closely linked to *nuc-2*. *nuc-2* and *preg*^{c} (constitutive) mutants are recessive to their wild-type alleles in partial diploids as well as in heterokaryons, while *pcon*^{c} mutants are dominant or co-dominant. *nuc-1* is epistatic to both *pcon*^{c} and *preg*^{c} mutants. The implications of these findings for theories of metabolic control in eukaryotes are briefly discussed.

THE synthesis of metabolically related enzymes in *Neurospora crassa* is under rigorous coordination. Studies of the control of enzyme synthesis have revealed a number of kinds of regulatory mutants (METZENBERG 1972). These regulatory mutants fall into two classes: "null" mutants that fail to make several metabolically related enzymes under conditions that permit their synthesis in wild type, and constitutive mutants, which make the enzymes under conditions that do *not* permit their synthesis in wild type. VALONE, CASE and GILES (1971) have identified a locus, *qa-1*, which controls the synthesis of three enzymes involved in the catabolism of quinic acid. "Null" mutants that are unable to make any of the three enzymes, and mutants that make all of them constitutively (*qa-1*^{c}) map very close to one another. These mutants are, in turn, very closely linked to the three structural genes, which are themselves clustered. Some of the "null" mutants are recessive in heterokaryons, while others are semi-dominant. Some constitutive mutants have also been examined in heterokaryons with their wild-type alleles. While none of the heterokaryons examined fails to show some constitutive synthesis of the enzyme that was examined, the levels of this enzyme ranged from 5% to 57% that of the constitutive member taken alone. It is not clear whether there is a product made at the site which gives rise to *qa-1*^{c} mutants.

POLACCO and GROSS (1973) have reported that mutations at the *leu-3* locus of Neurospora can give rise not only to mutants impaired in the synthesis of leucine

[1] This work was supported by an NIH grant, GM 08995.

[2] Supported by an NIH Career Development Award during most of the course of this research.

biosynthetic enzymes ("null" mutants) but also to conditional constitutive mutants. In this case, the control gene, *leu-3*, is not closely linked to the structural genes it controls. The dominance relationships in heterokaryons are complex; under some metabolic conditions, the constitutive mutant is approximately co-dominant, while under others it is essentially recessive. In any event, the *leu-3* gene product appears to exert positive control over several unlinked structural genes.

Positive control has also been implicated in the control of sulfur metabolism in Neurospora (MARZLUF and METZENBERG 1968; METZENBERG and AHLGREN 1971). The product of the *cys-3* gene is necessary for the expression of several unlinked structural genes. Another unlinked gene, *scon*, is the site of a constitutive mutation. *scon*^{c} (sulfur-control constitutive) is roughly co-dominant in heterokaryons with its wild-type allele. Surprisingly, *scon*^{c} appears to control only the synthesis of enzymes coded in the same nucleus (BURTON and METZENBERG 1972; DIETRICH and METZENBERG 1973). Thus in the sulfur system, both "null" mutants and constitutive mutants have been found, but not at the same locus.

Recently, we have described a Neurospora mutant constitutive for the synthesis of an alkaline phosphatase and a high-pH, high-affinity phosphate permease (LEHMAN *et al.* 1973). This mutant was roughly co-dominant in heterokaryons with its wild-type allele. The locus will be referred to herein as *pcon* (phosphorus control). The original allele, *pcon*$^{c-6}$, is very closely linked, and perhaps allelic with *nuc-2*, a "null" mutant that fails to make the enzymes of the phosphorus family, even under conditions of phosphorus starvation (TOH-E and ISHIKAWA 1971). A second, unlinked locus, *nuc-1*, gives rise to "null" mutants, but, in contrast to *nuc-2*, no constitutive mutations have been detected at this locus.

The dominance or recessiveness of "null" and constitutive mutants has heretofore been examined in heterokaryons with their wild-type alleles. The interpretation of such dominance studies has often been somewhat equivocal, for at least two reasons. First, the proportion of the two types of nuclei in a heterokaryon can vary widely, and the question of gene dosage in such a coenocyte must be carefully taken into account. Second, any gene product that is not freely diffusible between nuclei will affect only the nucleus that codes for it. In the present paper, we describe the preparation of heterozygous partial diploids, which circumvents some of the uncertainties involved in the interpretation of dominance studies with heterokaryons. We also describe the isolation of more *pcon*^{c} mutations, and of a new type of mutant constitutive for the synthesis of alkaline phosphatase, *preg*^{c}. The site of this mutation is quite close to that of *pcon*^{c}-*nuc-2*, but is nevertheless readily separable . *nuc-2* and *preg*^{c} are shown to be recessive to their wild-type alleles in partial diploids, and *pcon*^{c} is shown to be dominant or co-dominant.

MATERIALS AND METHODS

Media and growth conditions: Conditions for scoring for levels of alkaline phosphatase, scoring of *nuc-1* and *nuc-2*, conditions for crossing, and "repression" and "derepression" media have all been described previously (LEHMAN *et al.* 1973). In some of the experiments reported below,

derepression of alkaline phosphatase was produced by furnishing a phosphorus source (phosphoryl ethanolamine) that we have found to be used less readily than is inorganic phosphate, but which still allows growth at near maximal rates. This phosphate ester can be added to any desired concentration (generally 1–2 mM) without causing strong repression, so that large yields of rapidly-growing, derepressed cells may be obtained. The phosphate ester is filter-sterilized and added to autoclaved, phosphorus-free medium.

Extraction and assay of enzymes: These procedures have been described (LEHMAN *et al.* 1973).

Isolation of new constitutive mutants: This was done essentially as described by LEHMAN *et al.* (1973), with the staining procedure modified as described by TOH-E and ISHIKAWA (1971).

Strains and nomenclature: All the strains used were made heterokaryon-compatible with the two Oak Ridge wild types, 74-OR8-1a and 74-OR23-1A, and as nearly isogenic with them as practical by several sequential crosses to these standard strains. Most of the strains used in this study were obtained from the Fungal Genetics Stock Center (FGSC), and have been described previously (LEHMAN *et al* 1973). *nuc-1* is allele T28-M1 (FGSC #1994); *nuc-2* is allele T28-M2 (FGSC #1998). The original mutant constitutive for alkaline phosphatase and for a high affinity, high pH phosphate permease was originally called by its allele isolate number, UW-6 (LEHMAN *et al.* 1973). The locus at which this mutation occurs will henceforth be called *pcon*^{c} for "phosphorus-controller-constitutive", and the original (type) allele will be called *pcon*$^{c-6}$. Mutants at the *pcon*^{c} locus have already been shown to be co-dominant in heterokaryons with the wild-type allele, and it will be shown below that they are also co-dominant in heterozygous partial diploids. A second, rather closely linked locus (about 2.5 centimorgans to the right of *pcon*^{c}) is the site of a recessive mutation to constitutive formation of the alkaline phosphatase group of enzymes. This locus is called *preg*^{c} ("phosphorus-regulator-constitutive"). In the interest of clarity, we will prospectively call a given mutant "*pcon*^{c}" or "*preg*^{c}" in advance of presentation of mapping and dominance studies.

Stable diploids of the entire genome are unknown in Neurospora, but methods have been described for preparing partial diploids by crossing a strain bearing a chromosomal rearrangement to one without such a rearrangement (see, e.g., NEWMEYER and TAYLOR 1967; TURNER *et al.* 1969; PERKINS 1972). Asci in which duplication and deficiency progeny are segregating can be recognized by the fact that deficiency spores, which are lethal, remain white, while mature euploid and duplication-bearing spores are black. The duplication progeny are usually morphologically indistinguishable from wild type, but they can easily be identified by spotting conidial suspensions on lawns of mating-type tester strains (*fluffy*). When crossed to a tester of the opposite mating type, duplication-bearing strains produce approximately a normal number of perithecia, but very few ascospores. This trait is called Barren (TURNER *et al.* 1969). In such a test, the duplication strains studied in the present work gave approximately 10^{4}-fold fewer ascospores from roughly equal numbers of perithecia than did euploid Normal Sequence or translocation-bearing strains.

Duplication-bearing ascospores from a cross of a strain bearing an insertional translocation may also be unequivocally identified by using asci containing four white and four black spores (non-parental ditype asci). In the work reported here, the four viable, black spores have invariably proved to be duplication-bearing progeny, as judged by the Barren trait and by the segregation of linked markers; see, however, PERKINS (1972) and NEWMEYER and TAYLOR (1967).

With the rearrangements described by PERKINS (1972), it is now possible to cover about ⅓ to ½ of the Neurospora genome with duplications. Fortunately, *pcon*^{c} and *preg*^{c} are in such a region, as will be discussed in detail below. A euploid insertional translocation, T(II→I)NM177 (*A*, FGSC #1610 and *a*, FGSC #2003) has a piece of the right arm of linkage group II moved to linkage group I, just to the left of the mating type locus (shown by DR. ANNA KRUSZEWSKA. See PERKINS 1972). Crosses of this strain to Normal Sequence strains were used to generate all of the partial diploids used in this work.

For such partial diploids, the following nomenclature will be used: the two alleles will be given as superscripts, with the alleles to the left of the slant being on the Normal-Sequence

chromosome (Linkage Group II in this case), those to the right of the slant being on the translocated segment (in LG I, in this case).

GENETIC METHODOLOGY: PREPARATION AND CHARACTERIZATION OF PARTIAL DIPLOIDS

Preparation of putative partial diploids of the type pcon^c/pcon+ and preg^c/preg+: *pcon^{c-6} arom-1A* was crossed to T(II→I)NM177a, and ordered tetrads were obtained. Many of the asci contained either two or four white deficiency spores. An ascus with four white spores and four black spores (non-parental ditype) was dissected and the four black spores were germinated; as expected, the white spores were inviable. All four black spores were *arom-1*, consistent with the fact that *arom-1* is outside the segment covered by the duplication but rather close to the breakpoint in LG II; all four were also mating type *a*, which, in turn, is consistent with the fact that the translocation segment in its new location is closely linked to the mating type gene and to the left of it (PERKINS 1972). All four were also Barren, as expected.

Several non-parental ditype asci from the same cross were collected as unordered asci by the method of STRICKLAND (1960), as applied by PERKINS (1966a,b). The results were identical with those described above: all the black spores gave rise to *arom* cultures that were mating type *a* and Barren.

One of the markers known to be transposed in T(II→I)NM177 is *arg-12* (PERKINS 1972). We prepared partial diploids of the constitution *pcon^{c-6} arg-12/pcon+ arg-12+*; *inos* as follows. *pcon^{c-6} arg-12*; *inos-a* was crossed to T(II→I)NM177; *inos-A* and unordered asci were collected. An ascus containing four white and four black spores was examined as before. The four viable spores all gave rise to prototrophic, Barren cultures of mating type *A*.

preg^{c-1} arg-12/preg+ arg-12+ was prepared by dissection of unordered asci in a similar manner.

Preparation of nuc-2/nuc-2+ *partial diploids*: *nuc-2 arom-1A* was crossed to (T(II→I) NM177a and unordered asci were collected. An ascus containing four white and four black spores, and in which all four black spores germinated, was used to isolate the diploid. The four resulting cultures were all arom, mating type *a*, and Barren, as expected. *nuc-2 arg-12/nuc-2+ arg-12+* partial diploids were prepared in an analogous way.

Preparation of other partial diploids: A partial diploid in which both alleles at the *pcon* locus were wild type (*pcon+/pcon+*) was likewise prepared. Such a strain is also obviously *nuc-2+/ nuc-2+*

Partial diploids in which a *pcon^c* or *preg^c* allele was in the translocated segment (see below) and either a wild-type allele, or a different *pcon^c* or *preg^c* allele, or *nuc-2* were on the Normal Sequence LG II chromosome were prepared from unordered non-parental ditype asci. In each case, *arg-12* was present on the Normal Sequence LG II and *arg-12+* on the translocated segment. It was confirmed in each case that the putative diploids were of the expected mating type, and were Barren and arginine-independent.

Mapping of the mutations: The Normal Sequence gene order in LG II is centromere, *arg-5*, *pcon^c*, *arg-12*, *arom-1* (LEHMAN *et al.* 1973). Recombination between two Normal Sequence strains was examined by crossing constitutive strains with appropriate outside markers and plating heat-shocked random ascospores on non-selective medium with high phosphate. The plates were incubated for 2–3 days at 33° and irrigated with freshly-prepared, filter-sterilized stain. Unstained colonies were picked to culture tubes and allowed to conidiate. Conidial suspensions were streaked to check for possible heterokaryosis, and homokaryotic isolates were scored for outside markers, where these were present in the cross.

Examination of constitutive mutants in the translocation background T(II → I)NM177 for possible allelism was done by crossing two such translocation strains and looking for repressible segregants as described above. Since *arg-5* and *arom-1* are outside of the translocated segment, they cannot be used for mapping the constitutive mutants in the translocation strain. Neither can *arg-12* be used, since it has not yet been obtained in the T(II → I)NM177 background. However, the mating type locus, which is normally on LG I, is just to the right of the point of inser-

tion of the translocated segment. It proved to be closely linked to the constitutive mutants, and was used as an outside marker to establish the gene order.

Stability of the partial diploids: Some partial diploids in Neurospora are quite stable, while others rapidly lose part or all of the duplicated genetic material (PERKINS and NEWMEYER, personal communication; PERKINS, NEWMEYER and TURNER 1972). The dominance tests to be discussed below can only be interpreted if the partial diploids are reasonably stable.

To examine this point, conidia from a culture of *pcon^{c-6} arg-12/pcon+ arg-12+; inos-A* were plated onto BROCKMAN-DESERRES medium (see MATERIALS AND METHODS) supplemented with arginine and inositol. After 3 days at room temperature, the colonies were stained for repressible alkaline phosphatase. Among about 2070 colonies examined, none was completely repressible. Obviously *pcon^{c-6}* is dominant or co-dominant in partial diploids, as it is in heterokaryons. The eight most lightly stained colonies were picked and retested, and all were clearly derepressed. Thus the spontaneous rate of segregation of repressible colonies was too low to be detected in this experiment. Nevertheless, it seemed of value to see whether such segregation could be induced by ultraviolet light, especially since, if this occurred, it could be an aid in genetic analysis. Therefore a conidial suspension of the same strain was irradiated to 98.9% killing and was plated out as above. No repressed colonies were seen among about 580 examined. Thus there is no large percentage of *pcon+* haploids or *pcon+/pcon+* diploids segregating either spontaneously during vegetative growth, nor following irradiation. Obviously, this says nothing about whether *pcon^{c-6}* or *pcon^{c-6}/pcon^{c-6}* is segregating out, since these would not have been distinguished from *pcon^{c-6}/pcon+*.

A second test for breakdown of such a partial diploid is the segregation of argininеless auxotrophs, since the strain is heterozygous for *arg-12*. This was tested as follows. Conidia were plated on the medium of BROCKMAN and DE SERRES (1963) with inositol (50 μg/ml) and arginine (0.2 mM) to give 50–100 colonies per plate. (The arginine concentration was reduced fivefold from the usual 1 mM to decrease carryover to the replica plates.) The plates were incubated overnight at 33° and were replicated with sterile filter paper (LITTLEWOOD and MUNKRES 1972) to plates with inositol but without arginine. After two days, the plates were scored. Among 664 colonies, there were 4 arg auxotrophs (0.6%). These were picked from the master plate and saved.

Again, it seemed important to see if the frequency of segregation of argininеless auxotrophs from the partial diploid could be dramatically increased beyond the spontaneous rate. We attempted to do this by treating the strain with *p*-fluorophenylalanine, which, by little-understood mechanisms, causes haploidization and also vegetative recombination in *Aspergillus nidulans* (MORPURGO 1971; LHOAS 1961; SHANFIELD and KÄFER 1971). Conidia were inoculated to a concentration of 10^4/ml into Fries minimal medium plus arginine, inositol, and 0.1 mM DL-*p*-fluorophenylalanine. The suspension was agitated gently for 24 hours at room temperature. The conidia germinated but did not grow out into long hyphae in the presence of the analog. The suspension of germinated conidia was diluted 100-fold into 0.5 mM L-phenylalanine. Aliquots were plated and replicated as described above. Among 824 colonies, there were 10 arginine-requiring ones (1.2%), which is not significantly higher than the 0.6% that was found in the absence of treatment with *p*-fluorophenylalanine.

Testing of the 14 arginine-dependent vegetative segregants on plates containing high phosphate, arginine, and inositol showed that all still contained *pcon $^{c-6}$*. The strains were spotted on *fluffy-a* mating type tester plates (the *fluffy* strain was Normal Sequence). All were Barren, suggesting that all still bore a duplication.

While it was clear that arginine-requiring strains could be recovered from the partial diploid, it could still be argued that these might be new mutations, rather than segregants. This is especially true since the proportion of arginine-requiring strains is quite small, and arginine auxotrophs, which can occur at some 13 known loci, are among the most common auxotrophs. To test this, a heterokaryon was made with each of the 14 arginine-dependent isolates above and the strain *arg-12; inv; ars-101; al-2A*, using minimal sucrose plus arginine medium. *inos* and *inv* (invertaseless) were therefore the forcing markers. All of the heterokaryons grew very well on this medium, but none was able to grow when transferred to medium without arginine. Since

none of the strains complements with *arg-12*, the possibility that these are new arginine mutants (most of which would not be *arg*-12) can be discounted.

The mechanism of origin of the arginine-requiring strains was of interest with respect to behavior of the partial diploid. There were two obvious ways in which the *arg-12* segregants could have arisen. Double mitotic crossing over or gene conversion between the Normal Sequence chromosome bearing *pcon*$^{c-6}$ *arg-12* and the transposed segment bearing *pcon*+ *arg-12*+ could have given a partial diploid of the constitution *pcon*$^{c-6}$ *arg-12/pcon*$^{+}$ *arg-12*. On the other hand, part of the transposed segment bearing *arg-12*+ and perhaps *pcon*+ could have been simply deleted.

To distinguish between these possibilities, conidia of the arginine-dependent segregants were plated onto 80 cm^2 lawns of T(II→I)NM177; *fluffy a*. Despite the Barren phenotype, enough spores were produced from such crosses to allow a genetic analysis. Four such crosses were analyzed, two from arginine-dependent strains that arose spontaneously and two in which the segregation was "induced" by *p*-fluorophenylalanine. Since the results of all four were substantially identical, only those involving one of the spontaneously-arising segregants will be presented. Spores were heat-shocked and plated onto medium containing arginine and inositol. Among 39 cultures from this cross, 29 were arg+ a and 10 were arg A, showing complete linkage between *arg-12* and mating type, which are ordinarily unlinked. All of the arginine-dependent segregants carried *pcon*$^{c-6}$ and were Barren. Sixteen of the prototrophic segregants also carried *pcon*$^{c-6}$ and were Barren, while the other 13 prototrophic segregants were *pcon*+ and were fertile. The significant finding is the absence of a class: fertile *arg-12*, *pcon*$^{c-6}$ *A* strains. These would be expected to constitute about ¼ of all the progeny if the transposed segment were intact in the arginine-dependent vegetative segregants, but would be lethal deficiency strains if these segregants arose by interstitial deletion of part of the transposed segment carrying *arg-12*+. (See Figure 1 diagramming the expectations based on these two possibilities.) The results strongly

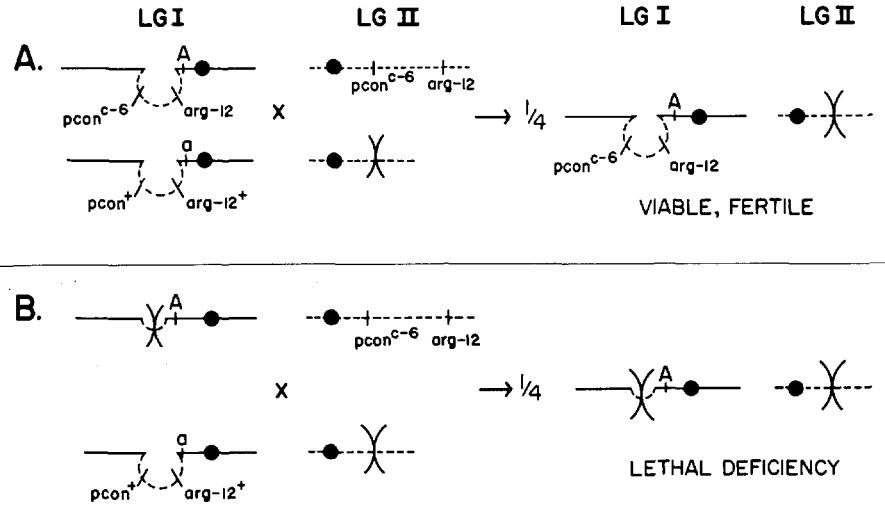

FIGURE 1.—Cross to determine the nature of arginine-requiring vegetative segregants. A. Expectation if arginine-requiring segregants are, in fact, *arg-12/arg-12* partial diploids resulting from double mitotic crossing over. B. Expectation if arginine-requiring segregants are haploid for *arg-12* due to deletion of part of the transposed segment carrying *arg-12*+. Material that is normally in Linkage Group I is depicted with straight lines, while that which is normally in Linkage Group II is depicted with a dashed line. Back-to-back parentheses)(indicate that a chromosomal segment has been deleted. Centromeres are shown as solid circles.

In panel A and in panel B, the euploid translocation strain is shown to the lower left quarter of the panel, and the two possible formulations for the arginine-requiring partial diploid are shown in the upper left quarter of the two panels.

argue that the arginine-dependent vegetative segregants arose by an interstitial deletion. It is noteworthy that such deletions do not seem to occur, at least not with comparable frequency, in the homologous segment in its normal location, since attempts to isolate *pcon*+ segregants from the duplication were unsuccessful. PERKINS, NEWMEYER and TURNER (1972) have discussed two other duplications in which only the transposed segment is lost, never the duplicated segment in its normal position. In his cases, unlike ours, fertility is restored. On the other hand, PERKINS and NEWMEYER have found that *most* deletions that remove the duplication of the mating type locus in an interstitial duplication (from strain T(I→II)39311) do not correct the Barren phenotype (personal communication). The same seems to be true of deletions of a terminal duplication produced by an inversion, In(IL→IR)H4250 (NEWMEYER, personal communication). Duplications involving T(II→I)NM177 show a combination of these two sets of properties: deletion appears to be specific to the transposed segment, but fertility is not restored.

The data, then, support the idea that conversion of the arginine-independent partial diploid to arginine-dependent form is associated with a deletion event. The simplest hypothesis is that part of the transposed segment, including the *arg-12*+ allele, is simply deleted, and with it perhaps the *pcon*+ allele. (The latter would not be detected with certainty in the "reduced" partial diploid, since *pcon*$^{c-6}$ is at least partially dominant in these partial diploids (see below).) However, it is possible to imagine that arginine dependence arises by a two-strand double mitotic crossover or conversion to give homozygosis rather than hemizygosis for *arg-12*, and perhaps, at the same time, homozygosis for *pcon*$^{c-6}$. Such an event might be associated with deletion of part of the transposed segment, but in such a way as to leave *pcon*$^{c-6}$ diploid and homozygous. Since *pcon*$^{c-6}$ on the transposed segment would be valuable both for mapping and for physiological studies, this possibility was explored. Conidia of the arginine-dependent "reduced partial diploid", mating type *A*, were spread on a lawn of Normal Sequence *fluffy a* bearing *arg-12*+ and *pcon*+, and the small yield of spores was isolated from the lid. If *pcon*$^{c-6}$ were present in the "reduced" translocated segment, almost all progeny that are mating type *A* will be either *pcon*$^{c-6}$/*pcon*$^{c-6}$, arginine-requiring, or *pcon*+/*pcon*$^{c-6}$ and arginine-independent; the exceptions will be rare crossovers between the mating type locus and the "reduced", translocated segment. In any event, both of these classes should be constitutive for alkaline phosphatase. Seven *A* progeny were obtained. Two of them were arginine-requiring and constitutive, and the other five were arginine-independent and were fully repressible. Both classes were Barren, as expected. Hence it seems clear that the "reduced partial diploid" is hemizygous, not homozygous, for *pcon*$^{c-6}$.

Attempts at getting pcon^{c-6} *into the translocated segment:* An attempt was made to introduce *pcon*$^{c-6}$ meiotically into the translocated segment by a two-strand double crossover. *arg-5 pcon*$^{c-6}$ *arom-1 A* was prepared by conventional means and was crossed to T(II→I)NM177 *fluffy*-a. *arg-5* and *arom-1* are flanking markers, outside of the region which is translocated (PERKINS 1972). Ascospores were germinated and spread on ten plates of minimal medium of BROCKMAN and DE SERRES (1963) to give about 3000 prototrophic colonies, and the plates were stained as usual. Two stained, *pcon*$^{c-6}$ colonies were found, and these were picked. One was *fluffy* and the other was not. If they were the result of double crossover events within the translocated segment, they should have been mating type *a*, but in fact, both were *A*. The mating type tests to Normal Sequence *fluffy* gave nearly 100% black spores. Hence it is clear that the *pcon*$^{c-6}$ prototrophic strains were not in the T(II→I)NM177 translocation. Apparently they arose by double crossovers between the shortened LG II chromosome of T(II→I)NM177 and the flanking markers on the Normal Sequence LG II. Other attempts to find double crossovers within the translocated region have been made, and these have been uniformly unsuccessful. Since the transposed region is rather short, it is quite possible that positive interference for double crossovers within this region is complete, or that the transposed segment does not pair with its homolog so as to allow recombination by gene conversion.

EXPERIMENTAL RESULTS

Isolation of new alkaline phosphatase-constitutive mutants in the T(II→I)-NM177 *background:* To establish that *pcon*$^{c-6}$ is really dominant or co-dominant to *pcon*+ it was essential to prove that the gene lies within the transposed segment

and is therefore heterozygous. It is known that *arg-12* lies within this segment (PERKINS 1972, and see above), and that *pcon*$^{c-6}$ is close to *arg-12* and to the left of it (LEHMAN *et al.* 1973). It is also clear that *nuc-2* is covered in the partial diploids (see below). However, since we were unable to transfer *pcon*$^{c-6}$ into LG I of *T(II→I)NM177* by recombination, it remained just possible that *pcon* was just to the left of *nuc-2* and left of the breakpoint in LG II of the translocation strain, albeit to the right of *arg-5*. If this were so, the putative partial diploids, *pcon*$^{c-6}$/*pcon*$^{+}$, would in fact be simply *pcon*$^{c-6}$.

We decided to isolate new constitutive mutants in the background of the insertional translocation and to determine their linkage. *T(II→I)NM177;inos-A* was irradiated with an ultraviolet sterilamp to about 90% killing and plated to give about 235 colonies per plate. (The inositol marker was included to facilitate later tests with forced heterokaryons.) Among about 18,750 colonies, there were four new constitutive mutants. They were purified by streaking conidia on plates, and were then outcrossed to *T(II→I)NM177-a*. Ascospores were plated to medium containing inositol, and germinated spores were picked. The resulting cultures were classified for mating type and for repressibility of alkaline phosphatase in the usual way. The results in Table 1 show that all four constitutive mutants in the translocation (T) background are closely linked to mating type, and are therefore in LG I in this strain. If they had not been in the translocated region, presumably they would have been found in LG II, as was the original *pcon*$^{c-6}$ strain.

Failure to find recombinants between pcon^{c-6} *and* nuc-2: In a previous paper, we reported that there were no wild-type recombinants in a cross between *pcon*$^{c-6}$ and *nuc-2* among 208 unselected progeny (LEHMAN *et al.* 1973). In order to obtain more extensive data, spores from crosses of these two mutants were plated under conditions which do not allow *nuc-2* sporelings to grow into colonies (see legend of Table 2) and the *nuc-2*$^{+}$ colonies were replicated to plates of high phosphate on which *pcon*$^{c-6}$ colonies will stain, but *pcon*$^{+}$ will not. As shown in Table 2, no *pcon*$^{+}$ *nuc-2*$^{+}$ colony was found among 854 *nuc-2*$^{+}$ colonies examined. This, of course, does not prove that the two mutants are allelic, but does show that they are at least very closely linked.

TABLE 1

Linkage to mating type of constitutive mutants in the translocation (T) background

Cross	Parental types: Repressible, m.t. *a*	Parental types: Constitutive, m.t. *A*	Recombinant types: Repressible, m.t. *A*	Recombinant types: Constitutive, m.t. *a*
(T)pcon^{c-2} *A* × *(T)pcon*$^{+}$ *a*	275	N.D.	N.D.	2
(T)pcon^{c-3} *A* × *(T)pcon*$^{+}$ *a*	33	31	1	2
(T)pcon^{c-7} *A* × *(T)pcon*$^{+}$ *a*	54	N.D.	N.D.	1
(T)preg^{c-1} *A* × *(T)preg*$^{+}$ *a*	134	144	3	2

Progeny from the crosses were scored on spot plates for repressibility of synthesis of alkaline phosphatase and for mating type. In two of the crosses, only strains that were mating type *a* were saved and scored for repressibility, and the *A* progeny phenotypes were not determined (N.D.).

TABLE 2

Lack of recombination between pcon^{c-6} *and* nuc-2

Cross			*nuc-2*+ colonies examined	*pcon*+ *nuc-2*+ colonies
arg-5	*pcon*$^{c-6}$	*arg-12*+		
	×		465	0
arg-5+	*nuc-2*	*arg-12*		
arg-5	*pcon*$^{c-6}$	*arom-1*+		
	×		389	0
arg-5+	*nuc-2*	*arom-1*		

Spores from these two crosses were plated to a medium containing the sugar mixture of BROCKMAN and DE SERRES (1963) and low phosphate (0.05 mM) with the pH maintained at pH 7 with 0.1 M Na-MOPS buffer (LEHMAN *et al.* 1973). Under these conditions, *nuc-2* does not grow. In the first cross above, the medium was also supplemented with arginine; in the second cross, with arginine plus the four aromatic amino acids. The colonies were then replicated (LITTLEWOOD and MUNKRES 1972) to supplemented Fries BROCKMAN-DE SERRES plates containing the normal (high—7.35 mM) level of phosphate. The colonies on the master and replica plates were stained for alkaline phosphatase. *pcon*+ *nuc-2*+ colonies would have been detected by their ability to stain on low phosphate and their failure to stain on high phosphate. None was found; all colonies were stained on both the master and replica plates.

Crosses between Normal Sequence pcon^{c} *and* preg^{c} *mutants:* The "type" strains, *pcon*$^{c-6}$ and *preg*$^{c-2}$, were crossed. Random ascospores were plated to minimal (high phosphate) BROCKMAN-DE SERRES medium. The plates were incubated and the colonies were stained for alkaline phosphatase in the usual way. Of 815 colonies, 9 were unstained (repressible). If the double mutant is assumed to be non-repressible as are the two parental strains, the recombination frequency can be estimated as about 2%.

To establish the gene order of *pcon*$^{c-6}$ and *preg*$^{c-2}$ on LG II, a similar cross was made with strains bearing outside markers. *arg-5 preg*$^{c-2}$ was crossed to *pcon*$^{c-6}$ *arom-1*. Random spores were plated to medium as above, supplemented with arginine and the four aromatic amino acids. The resulting colonies were stained aseptically for alkaline phosphatase. Among 2780 colonies, 15 were unstained (0.54%). These were picked to supplemented medium, and were scored on spot plates for nutritional requirements. The results in Table 3 show that, among repressible recombinants, most are *arg-5* and *arom-1*. From this, the gene order is inferred to be *arg-5 pcon preg arom-1*.

In a confirmatory experiment, *arg-5 pcon*$^{c-6}$ was crossed to *preg*$^{c-2}$ *arg-12*. Random ascospores were plated to arginine-supplemented medium, and the resulting colonies were stained. Of 836 colonies examined, only one was unstained, and it proved to be a prototroph. This is consistent with the expected gene order *(centromere) arg-5 pcon preg arg-12 (arom-1)*.

A group of newly isolated constitutive mutants were found to be dominant in partial diploids. They were therefore suspected of being alleles of *pcon*$^{c-6}$. These new mutants were crossed to *pcon*$^{c-6}$ *arg-12* and to *preg*$^{c-2}$ *arg-12*. Ascospores were plated to arginine-supplemented medium, and the colonies were stained as above. The results are presented in Table 4. All of them give very roughly 1%

TABLE 3

Gene order of pcon^{c-6} *and* preg^{c-2}

Inferred gene order

arg-5	*pcon+*	*preg^{c-2}*	*arom-1+*
region 1	region 2	region 3	
arg-5+	*pcon^{c-6}*	*preg+*	*arom-1*

Class of segregant	Genotypes	Number
Constitutive	*arg-5 pcon+ preg^{c-2} arom-1+* parent; *arg-5+ pcon^{c-6} preg+ arom-1* parent; and probably some *pcon^{c-6}*, *preg^{c-2}* recombinants	2767
Repressible; Crossovers in Region 2	*arg-5 pcon+ preg+ arom-1*	13
Repressible; Crossovers in Regions 2 and 1	*arg-5+ pcon+ preg+ arom-1*	1
Repressible; Crossovers in Regions 2 and 3	*arg-5 pcon+ preg+ arom-1+*	1

The parental strains were *pcon^{c-6}* and *preg^{c-2}* with outside markers as shown above. Repressible recombinants were screened as described in the text.

TABLE 4

Non-allelism of new (putative pcon^{c}*) constitutive mutants with* preg^{c-2} *and probable allelism with* pcon^{c-6}

	Cross to *pcon^{c-6} arg-12*		Cross to *preg^{c-2} arg-12*	
New mutant	Total colonies	Unstained	Total colonies	Unstained
pcon^{c-102}	1077	0	590	4
pcon^{c-112}	1101	0	827	11
pcon^{c-120}	736	2	1190	14
pcon^{c-121}	1044	0	739	7
pcon^{c-136}	603	0	871	4
pcon^{c-145}	1377	0	332	5

The conditions of the experiment are described in the text. All of the unstained, repressible cultures proved to be *arg-12+*. This is consistent with the gene order inferred in the previous table.

repressible recombinants in crosses with *preg^{c} arg-12*. In crosses of the new mutants to *pcon^{c-6} arg-12*, *pcon^{c-120}* gave 2 repressible colonies among 736 colonies; both of these were *arg-12+*. The rest of the crosses to *pcon^{c-6} arg-12* gave no repressible recombinants among 600 to 1300 colonies.

Crosses between Translocation (T) strains bearing constitutive mutations: The four mutants which were isolated in the *T(II→I)NM177A* background were obtained in mating type *a* (see Table 1). (T)*pcon^{c-2} A* was crossed to (T)*pcon^{c-3} a* and to (T)*pcon^{c-7} a*. Spores were plated onto BROCKMAN-DE SERRES medium (high phosphate) and the resulting colonies were stained as usual. As noted in Table 5, there were no repressible recombinants among the segregants examined.

TABLE 5

Crosses between constitutive mutants in the translocation (T) background

Cross	Total colonies	Repressible recombinants
(T)*pcon*$^{c-2}$ *A* × (T)*pcon*$^{c-3}$ *a*	1351	0
(T)*pcon*$^{c-2}$ *A* × (T)*pcon*$^{c-7}$ *a*	916	0
(T)*preg*$^{c-1}$ *A* × (T)*pcon*$^{c-3}$ *a*	1109	13; all m.t. *a*
(T)*preg*$^{c-1}$ *A* × (T)*pcon*$^{c-7}$ *a*	1044	10; all m.t. *a*

The conditions of the experiment are described in the text.

On the other hand, a cross of (T)*preg*$^{c-1}$ *A* to either (T)*pcon*$^{c-3}$ *a* or (T)*pcon*$^{c-7}$ *a* gave roughly 1% repressible colonies in each case. Each of the repressible colonies was picked and purified by streaking conidia. All of the 23 repressible recombinants isolated from these two crosses proved to be mating type *a*. Since the translocated segment is to the left of mating type in LG I, the following order of genes can be inferred: *pcon, preg, mating type, (centromere)*. Thus the translocated segment is probably oriented with respect to its new centromere in the opposite polarity to its orientation with respect to the centromere in Normal Sequence.

Crosess between Normal Sequence preg^{c} *and* nuc-2: *arg-5 preg*$^{c-2}$ was crossed to *nuc-2 arom-1* and ascospores were plated to medium supplemented with arginine and the four aromatic amino acids, with low phosphate at pH 7 so that only *nuc-2*$^{+}$ sporelings were able to grow. Colonies were replicated as described in the legend of Table 2, and the master and replica plates were stained aseptically. The results are summarized in Table 6. It can be seen that, among the 11 recombinants that are able to make alkaline phosphatase on low phosphate but not on high phosphate (*nuc-2*$^{+}$, *preg*$^{+}$), 10 are *arg-5 arom-1*. This is consistent with the previous indications that *nuc-2* is very closely linked, and perhaps allelic with *pcon*, and *pcon* is to the left of *preg*.

TABLE 6

Gene order of nuc2 *and* preg^{c-2}

Cross				*nuc-2*$^{+}$ colonies examined	*nuc-2*$^{+}$, *preg*$^{+}$ colonies	
arg-5	*nuc-2*+	*preg*$^{c-2}$	*arom-1*+			
		×		365		
arg-5+	*nuc-2*	*preg*+	*arom-1*		1 *arg-5*	*arom-1*+
arg-5	*nuc-2*	*preg*+	*arg-12*+		20 *arg-5*+	*arg-12*+
		×		748		
arg-5+	*nuc-2*+	*preg*$^{c-2}$	*arg-12*		2 *arg-5*	*arg-12*+

The experiments were performed as described in the legend of Table 2. The two repressible recombinants in the second cross that were auxotrophs were identified as *arg-5* by their ability to complement in heterokaryons with an *arg-12* tester strain and their inability to complement with *arg-5*.

In a confirmatory cross, *arg-5 nuc-2* was mated to *preg*$^{c-2}$ *arg-12* and the spores were plated as above, except that the aromatic amino acids were omitted from the medium. Again, replica plates were prepared, and the master and replica plates were stained. Among the 22 repressible recombinants, 20 were *arg-5*$^+$, *arg-12*$^+$, which again is consistent with the postulated gene order.

Recessiveness of nuc-2 *in partial diploids:* It is known that *nuc-2* is recessive in heterokaryons (TOH-E and ISHIKAWA 1971). This appears to rule out the possibility that *nuc-2* makes a superrepressor that is available to all nuclei in the heterokaryon, but does not exclude the possibility that it makes a nucleus-restricted superrepressor. This question can be examined with partial diploids, in which both alleles of *nuc-2* are present within the same nuclear membrane. The results in Table 7 show that *nuc-2* is virtually completely recessive to its wild-type allele in partial diploids as well. The differences between the heterozygous diploids and *nuc-2*$^+$ haploid and the *nuc-2*$^+$/*nuc-2*$^+$ diploid control stains are not significant (t-test).

Dominance or co-dominance of pcon^c *mutants in partial diploids:* It was reported previously that the original isolate at this locus (*pcon*$^{c-6}$ or UW-6: LEHMAN *et al.* 1973) is roughly co-dominant in heterokaryons. The data in Table 8 show that this and various other putative alleles at this locus were likewise co-dominant or dominant in partial diploids with their wild-type allele. In the case of *pcon*^{c} alleles 2 and 7 that were isolated in the translocation strain, it was convenient to make partial diploids in which the Normal Sequence chromosome carried *nuc-2* as well.* It is obvious from Table 8 that such *pcon*$^+$*nuc-2*/*pcon*^{c}*nuc-2*$^+$ strains are constitutive.

TABLE 7

Recessiveness of nuc-2 *in partial diploids*

Control strains and partial diploids	Alkaline phosphatase, specific activity ± S.E.	
	Repression medium, P_i = 7.35 mM	Derepression medium, P_i = 0.05 mM
nuc-2	1.00	1.12
wild type (*nuc-2*$^+$)	1.43 ± 0.052 (N=5)	1038 ± 37 (N=2)
arom-1	1.10	807
nuc-2$^+$/*nuc-2*$^+$	1.80	922
nuc-2 arom-1/*nuc-2*$^+$	1.01 ± 0.05 (N=4)	727 ± 152 (N=4)
nuc-2 arg-12/*nuc-2*$^+$ *arg-12*$^+$	1.67 ± 0.47 (N=4)	965 ± 50 (N=20)

All of the strains were grown by sparging with air for 15 hours at 25° in Fries medium with high or low phosphate. The *arom* strains were grown on the same media supplemented with L-tryptophan, L-tyrosine, L-phenylalanine, and *p*-aminobenzoic acid (0.5, 0.5, 0.25 and 0.005 mM, respectively).

The wild-type figures represent separate growth flasks of the same strain, 74-OR8-1a. The three cases in which N=4 represent cultures from the four partial diploid spores of a single non-parental ditype ascus. The case in which N=20 represents cultures from all partial diploid spores of five such asci.

* Since the possible allelism of *pcon*^{c} with its "null" counterpart, *nuc-2*, has not been established beyond all doubt, we will conservatively use the notation *pcon*$^+$ *nuc-2*/*pcon*^{c} *nuc-2*$^+$; however, it remains quite possible that *nuc-2* occurs in the same cistron as *pcon*^{c}, in which case *pcon* would be a synonym for *nuc-2*, and the heterozygote could be written *pcon*/*pcon*^{c}.

TABLE 8

Dominance or co-dominance of pcon^c *alleles in partial diploids on repression medium*

Haploids	Alk. p'ase sp. act.	Diploids with *pcon+*	sp. act. Alk. p'ase
pcon+ (wild type)	1.52±0.19 (N=5)	*pcon+/pcon+*	1.32
pcon^{c-6}	150	*pcon^{c-6} arg-12/pcon+ arg-12+*	225±12.4 (N=4)
pcon^{c-102}	720	*pcon^{c-101}/pcon+*	351
pcon^{c-112}	390	*pcon^{c-112}/pcon+*	550
pcon^{c-120}	143	*pcon^{c-120}/pcon+*	61.7
pcon^{c-121}	219	*pcon^{c-121}/pcon+*	331
(T)*pcon+*	1.42		
(T)*pcon^{c-2}*	567	*pcon+ arg-12/pcon^{c-2} arg-12+*	249±10 (N=12)
(T)*pcon^{c-7}*	312	*pcon+ arg-12/pcon^{c-7} arg-12+*	38.8±2.1 (N=12)
		Diploids with *pcon^{c-6}*	
		pcon^{c-6} arg-12/pcon^{c-2} arg-12+	351±11 (N=13)
		pcon^{c-6} arg-12/pcon^{c-7} arg-12+	285±7.2 (N=12)
		Diploids with *nuc-2*	
		pcon+ nuc-2 arg-12/pcon^{c-2} nuc-2+ arg-12+	398±25 (N=12)
		pcon+ nuc-2 arg-12/pcon^{c-7} nuc-2+ arg-12+	28.3±5.7 (N=12)

The strains were grown in stationary flasks for 3 days at 25° in Fries medium with high (7.35 mM) phosphate. The mycelial pads were harvested, washed, and enzyme extracts were made and assayed in the usual way. The specific activities, once again, are given with standard errors for those instances in which a number of cultures were run. In the case of wild type, quintuplicate flasks were grown and assayed. In the case of most of the rest, all the diploid strains derived from one or several asci were examined.

In the case of the *pcon*^{c} mutants isolated in the translocation strain, partial diploids with *pcon*$^{c-6}$ were prepared. As expected, these were also constitutive (Table 8).

A third *pcon*^{c} allele in the translocation background and two more in the Normal Sequence background have been examined for dominance in an identical manner. These results are omitted from Table 8 in the interest of brevity, since they are not notably different from the ones that are reported. In addition, all of the strains were routinely examined for alkaline phosphatase levels under conditions of derepression—in this case, with 1 mM or 2 mM phosphoryl ethanolamine as sole phosphorus source. As expected, the derepressed levels of enzyme were high, in each case, and not significantly different from that of derepressed wild type. These data are omitted as well.

At least one representative of each strain has been checked for repressibility in sparged exponentially growing culture as well. The specific activity of alkaline phosphatase under these conditions is almost always lower (about 2–3 fold) than in stationary cultures grown for 3 days, but the results are otherwise very similar (data not included).

Recessiveness and complementation of preg^{c} *mutants in partial diploids:* The data in Table 9 show that the two *preg*^{c} strains are recessive to the wild-type allele in partial diploids. In the case of (T)*preg*$^{c-1}$ it was possible to test this in a diploid with *nuc-2*, the two mutants being in repulsion. Such strains proved to be repressible. This is not surprising, since the map distance between these loci suggests that they should be separate cistrons, and since both are recessive to their wild-type alleles. As expected from the fact that *pcon*^{c} mutants are dominant, a partial diploid of the constitution *pcon*$^{c-6}$*preg*$^{+}$/*pcon*$^{+}$*preg*$^{c-1}$ is constitutive. Since *preg*$^{c-2}$ and (T)*preg*$^{c-1}$ are both recessive, it is significant that they do *not* complement with one another in *preg*$^{c-2}$/*preg*$^{c-1}$ to give a repressible phenotype. This adds credibility to the idea that they are alleles.

To assess the stability of heterozygous partial diploids containing *preg*^{c} mutations, *preg*$^{c-2}$/*preg*$^{+}$ and *preg*$^{+}$/*preg*$^{c-1}$ conidia were plated to minimal (high phosphate) BROCKMAN-DE SERRES medium to give about 50 colonies per plate. The plates were incubated for three days at 25° and stained for alkaline phosphatase. *preg*$^{c-2}$/*preg*$^{+}$ gave 5 derepressed colonies among 974 examined; this rate of breakdown is in good agreement with that found earlier for elimination of *arg-12*$^{+}$ from *arg-12/arg-12*$^{+}$ partial diploids. *preg*$^{+}$/*preg*$^{c-1}$ gave one derepressed colony among 1291 examined. It is not known whether this single segregant represents deletion of the wild-type allele from the Normal Sequence chromosome, or mitotic crossing over, or a new mutation. In any event, it is clear that these diploids are not breaking down at a rate that is high enough to vitiate the results of dominance tests.

Recessiveness of preg^{c-2} *in a heterokaryon:* If the *preg*$^{+}$ product diffuses freely through the cytoplasm, one would expect *preg*^{c} to be recessive in a heterokaryon as it is in partial diploids. However, it is quite possible to imagine that the *preg*$^{+}$ product might be limited to the nucleus in which it was produced, or to the vicinity of that nucleus (see BURTON and METZENBERG 1972 for an example of

TABLE 9

Recessiveness of preg^{c} *alleles in partial diploids on repression medium*

Haploids	Alk. p'ase sp. act.	Diploids	Alk. p'ase sp. act.
preg+ (wild type)	1.52±0.19 (N=5)	*preg*+/*preg*+	1.32
preg^{c-2}	600	*preg*$^{c-2}$/*preg*+	1.27±0.03 (N=12)
(T)*preg*$^{c-1}$	521	*preg*+ *arg-12*/*preg*$^{c-1}$ *arg-12*+	1.47±0.07 (N=9)
		preg^{c-2}/*preg*$^{c-1}$	423±44 (N=12)
		pcon^{c-6} *preg*+ *arg-12*/*pcon*+ *preg*$^{c-1}$ *arg-12*+	144±11 (N=10)
		nuc2 preg+ *arg-12*/*nuc-2*+ *preg*$^{c-1}$ *arg-12*+	0.98±0.09 (N=12)

The experiment was done as described in the legend of Table 8.

such a limitation). If this were the case, *preg*$^{c-2}$ might be co-dominant in a heterokaryon. A nutritionally forced heterokaryon was prepared between *preg*$^{c-2}$ *arg-12* and *inos; albino-2*. This heterokaryon was grown on Fries minimal medium under the same conditions as noted in the legend of Table 9, and an extract of the heterokaryon was assayed for alkaline phosphatase. The specific activity was 1.84, which is not markedly different from that of a wild-type homokaryon. The proportion of the two nuclear types in the balanced heterokaryon was estimated on selective plates by the general method of ATWOOD and MUKAI (1955). The frequency of *preg*$^{c-2}$ *arg-12* nuclei was found to be 33.8%. If *preg*$^{c-2}$ were co-dominant with its wild-type allele, a specific activity of roughly 203, or 33.8% of 600, would have been expected (Table 9). A comparable experiment with (T)*preg*$^{c-1}$ shows that it, too, is recessive (data not reported). Since *preg*$^{+}$ is dominant in heterokaryons, it appears that its product is not limited to the nucleus in which it is made.

Epistasis of nuc-1 *to* preg^{c-2}, *(T)*preg^{c-1}, *and (T)*pcon^{c-2}: We have reported that *nuc-1* is epistatic to *pcon*$^{c-6}$ (LEHMAN *et al.* 1973). To see whether *nuc-1* is also epistatic to the *preg*^{c} mutants, and also to a *pcon*^{c} mutant in the translocated position, double mutants were constructed as follows. *nuc-1* was crossed to *preg*$^{c-2}$ and 11 unordered tetrads were dissected. The progeny were scored on BROCKMAN-DE SERRES spot plates with low and high phosphate. There were three parental ditype asci, eight non-parental ditype asci, and no tetratypes. The latter is not surprising, since *nuc-1* is quite closely linked to the centromere of LG I (HASUNUMA and ISHIKAWA 1972). An isolate from one of the non-parental ditype asci which did not stain on low or high phosphate (viz., a putative *nuc-1; preg*$^{c-2}$ double mutant) was outcrossed to wild type and ascospores were plated to high phosphate BROCKMAN-DE SERRES medium. The resulting colonies were stained to detect alkaline phosphatase. As expected, roughly 1/4 of the colonies were constitutive. Hence, the genotype of the putative double mutant was confirmed, and *nuc-1* is epistatic to *preg*$^{c-2}$.

Preparation of (T)*preg*$^{c-1}$ *nuc-1* required a crossover rather than independent assortment, since the two genes are on the same chromosome. (T)*preg*$^{c-1}$ *arg-12*$^{+}$*A* was crossed to Normal Sequence *nuc-1; preg*$^{+}$ *arg-12* and ascospores were plated to minimal BROCKMAN-DE SERRES medium so that only (T)*arg-12*$^{+}$ and partial diploid *arg-12/arg-12*$^{+}$ sporelings grew into colonies. Twenty-eight colonies were picked and tested on spot plates of low phosphate. Of these, two did not stain. One was mating type *a* and was Barren. It can be assumed to be the diploid, *preg*$^{+}$ *arg-12 nuc-1a/preg*$^{c-1}$ *arg-12*$^{+}$. The other non-stainer was mating type *A*, fertile, and did not give an appreciable number of white spores in subsequent crosses with a translocation strain. It can be designated as (T)*preg*$^{c-1}$ *nuc-1 A*. If *nuc-1* were not epistatic to *preg*$^{c-1}$, it should have been impossible to isolate a translocation-bearing strain that failed to make alkaline phosphatase both on low phosphate and on high phosphate plates.

(T)*pcon*$^{c-2}$ *nuc-1* was prepared in a similar manner. Once again, the fact that a strain with the above properties could be isolated shows that *nuc-1* is epistatic in this case, as in others.

DISCUSSION

nuc-1 is recessive to its wild-type allele in heterokaryons (ISHIKAWA *et al.* 1969, and confirmed by us) and in partial diploids (METZENBERG—to be published). The results presented in this paper show that it is epistatic to both kinds of constitutive mutants, *pcon*^{c} and *preg*^{c}. Therefore, the product of the *nuc-1*$^+$ gene appears to be necessary, but not sufficient for, the synthesis of repressible alkaline phosphatase (and some other enzymes of phosphorus metabolism).

nuc-2 is likewise recessive in heterokaryons and in diploids. Taken alone, the fact that it is recessive to its wild-type allele in heterokaryons argues that *nuc-2* cannot be making a freely diffusible superrepressor (WILLSON *et al.* 1964). However, it does not exclude the possibility that such a superrepressor is made by *nuc-2* but is limited to its own nucleus. This possibility is effectively ruled out by the fact that *nuc-2* is also recessive in diploids. One possible interpretation is that *nuc-2*$^+$ codes for a positive control element that is necessary for expression of the structural genes of alkaline phosphatase (and its congeners); *nuc-2* can be viewed as an inactive allele. The observation that heterozygotes of the type *pcon*$^+$ *nuc-2*/*pcon*^{c}*nuc-2*$^+$ are constitutive is also in harmony with this interpretation.

In a similar vein, our previous finding that heterokaryons of *pcon*$^{c-6}$ with *pcon*$^+$ are constitutive, or at least not fully repressible, could be interpreted in more than one way. One could suppose that the *presence* of a *pcon*^{c} allele in a particular nucleus, along with *nuc-1*$^+$ and *nuc-2*$^+$, causes a strain to be constitutive. On the other hand, one could postulate that the *absence* of a *pcon*$^+$ allele in a particular nucleus causes a strain to be constitutive, with the product of the *pcon*$^+$ allele being limited to the nucleus that codes for it. Since heterozygous *pcon*^{c}/*pcon*$^+$ and *pcon*$^+$/*pcon*^{c} diploids are constitutive, the second possibility can be rejected. Hence, *pcon*$^+$ cannot code for a repressor, not even a nucleus-limited one. It seems likely that *pcon*$^+$ represents a control region, presumably a receptor for a negative regulator of synthesis of alkaline phosphatase and its congeners. *pcon*^{c} mutants are viewed as having lost some or all of their affinity for this regulator. The data in this paper do not afford any insight as to whether *pcon* is a receptor on the chromosome (i.e., an operator) or a receptor on an RNA, protein, or other macromolecule.

The recessiveness and map position of *preg*^{c} mutants argues that the *preg* gene is functionally distinct from the *pcon* gene. *preg*^{c} is recessive to *preg*$^+$ in both diploids and in heterokaryons. We suggest the hypothesis that *preg*$^+$ makes a diffusible regulatory product that is *formally* identical with a repressor or a corepressor. It could work on an operator at the level of transcription, but could equally well exert its effects on a macromolecule other than DNA.

If *pcon*$^+$ is an operator, one might suppose that *nuc-2*$^+$ is under its control. In the presence of corepressor (phosphate?) and repressor (*preg*$^+$ product?) *nuc-2*$^+$ product would not be made, and expression of the (presumably unlinked) structural genes would not occur. In the absence of either corepressor or repressor, *nuc-2*$^+$ product would be made, and, if *nuc-1*$^+$ product is also present, the structural genes would be expressed.

An alternative model is that the *pcon* locus does give rise to a gene product, and that *pcon* and *nuc-2* are in reality a single cistron that codes for a bifunctional product. This product is required for transcription or translation of the structural genes, and its activity is abolished when *preg*$^+$ product is bound to it. In *pcon*^{c} mutants, the binding site for *preg*$^+$ product is altered or abolished, and in *nuc-2* the "activity site" necessary for expression of the structural genes is altered or abolished.

Both of these models predict that if a *pcon*^{c} *nuc-2* double mutant could be constructed and put into a diploid with *pcon*$^+$ *nuc-2*$^+$, such a cis diploid would have very different behavior from that of a trans diploid, which is constitutive (Table 8). In some other respects, the predictions of the two models are not identical. For example, the second model suggests that a single mutation might affect both the ability to express the structural genes on low phosphate ("*nuc-2* function") and the ability of high phosphate to repress ("*pcon* function"). Some tests of these predictions are in progress.

The map distance between *pcon* and *preg* (about 2 centimorgans) poses two questions: why is it so large? and, why is it so small? This recombination frequency seems too great for these two to be in the same cistron, or even in neighboring cistrons (see STADLER and TOWE 1968; MURRAY 1970; RINES, CASE and GILES 1969; DE SERRES 1969 for some representative intracistron and intercistron map distances in Neurospora). While it is possible that this is a recombinational "hot spot", the fact that recombination between *pcon* and *preg* always seems to be accompanied by recombination of outside markers suggests that most or all of the repressible recombinants are the result of classical crossing over, not gene conversion. On the other hand, two centimorgans is an intriguingly small distance. The total genome of *Neurospora crassa*, which consists of seven chromosomes, is hundreds of centimorgans long. It is certainly possible that *pcon* and *preg* are rather closely linked for purely accidental reasons, but the possibility that there is some causal explanation cannot be ignored.

The authors are grateful for the fine technical assistance of MR. DAVID BESWICK in recent phases of this work, and of Ms. CIDIA MEI-SHENG LU during an earlier period. They are especially indebted to DRS. DOROTHY NEWMEYER and DAVID PERKINS for a critical reading of the manuscript.

LITERATURE CITED

ATWOOD, K. C. and F. MUKAI, 1955 Nuclear distribution in conidia of Neurospora heterokaryons. Genetics **40**: 438–443.

BROCKMAN, H. E. and F. J. DE SERRES, 1963 "Sorbose toxicity" in *Neurospora*. Am. J. Botany **50**: 709–714.

BURTON, E. G. and R. L. METZENBERG, 1972 Novel mutation causing derepression of several enzymes of sulfur metabolism in *Neurospora crassa*. J. Bacteriol. **109**: 140–151.

DE SERRES, F. J., 1969 Comparison of the complementation and genetic maps of closely linked nonallelic markers on linkage group I of *Neurospora crassa*. Mutation Res. **8**: 43–50.

DIETRICH, P. S. and R. L. METZENBERG, 1973 Metabolic suppressors of a regulatory mutant in *Neurospora*. Biochem. Genet. **8**: 73–84.

HASUNUMA, K. and T. ISHIKAWA, 1972 Properties of two nuclease genes in *Neurospora crassa*. Genetics **70**: 371–384.

ISHIKAWA, T., A. TOH-E, I. UNO and K. HASUNUMA, 1969 Isolation and characterization of nuclease mutants in *Neurospora crassa*. Genetics **63**: 75–92.

LEHMAN, J. F., M. K. GLEASON, S. K. AHLGREN and R. L. METZENBERG, 1973 Regulation of phosphate metabolism in *Neurospora crassa*. Characterization of regulatory mutants. Genetics **75**: 61–73.

LHOAS, P., 1961 Mitotic haploidization by treatment of *Aspergillus niger* diploids with *para*-fluoro-phenylalanine. Nature **190**: 744.

LITTLEWOOD, R. K. and K. D. MUNKRES, 1972 Simple and reliable method for replica plating in *Neurospora crassa*. J. Bacteriol. **110**: 1017–1021.

MARZLUF, G. A. and R. L. METZENBERG, 1968 Positive control by the *cys-3* locus in regulation of sulfur metabolism in *Neurospora*. J. Mol. Biol. **33**: 423–437.

METZENBERG, R. L., 1972 Genetic regulatory mechanisms in *Neurospora*. Ann. Rev. Genet. **6**: 111–132.

METZENBERG, R. L. and S. K. AHLGREN, 1971 Structural and regulatory control of aryl sulfatase in *Neurospora*: the use of interspecific differences in structural genes. Genetics **68**: 369–381.

MORPURGO, G., 1961 Somatic segregation induced by *p*- fluorophenylalanine. Aspergillus Newsl. **2**: 10.

MURRAY, N. E., 1970 Recombination events that span sites within neighbouring gene loci of *Neurospora*. Genet. Res. **15**: 109–121.

NEWMEYER, D. and C. W. TAYLOR, 1967 A pericentric inversion in Neurospora, with unstable duplication progeny. Genetics **56**: 771–791.

PERKINS, D. D., 1966a Details for collection of asci as unordered groups of eight projected ascospores. Neurospora Newsl. **9**: 11. ——, 1966b Preliminary characterization of chromosome rearrangements using shot asci. Neurospora Newsl. **9**: 10–11. ——, 1972 An insertional translocation in Neurospora that generates duplications heterozygous for mating type. Genetics **71**: 25–51.

PERKINS, D. D., D. NEWMEYER and B. C. TURNER, 1972 Nontandem duplications in Neurospora, and restoration of the euploid condition by chromosome breakage. Genetics **71**: s46–s47 (Abst.).

POLACCO, J. C. and S. R. GROSS, 1973 The product of the *leu-3* cistron as a regulatory element for the production of the leucine biosynthetic enzymes of Neurospora. Genetics **74**: 443–459.

RINES, H. W., M. E. CASE and N. H. GILES, 1969 Mutants in the *arom* gene cluster of *Neurospora crassa* specific for biosynthetic dehydroquinase. Genetics **61**: 789–800.

SHANFIELD, B. and E. KÄFER, 1971 Chemical induction of mitotic recombination in *Aspergillus nidulans*. Genetics **67**: 209–219.

STADLER, D. R. and A. M. TOWE, 1968 A test of coincident recombination in closely linked genes of Neurospora. Genetics **58**: 327–336.

STRICKLAND, W. N., 1960 A rapid method of obtaining unordered Neurospora tetrads. J. Gen. Microbiol. **22**: 583–588.

TOH-E, A. and T. ISHIKAWA, 1971 Genetic control of the synthesis of repressible phosphatases in *Neurospora crassa*. Genetics **69**: 339–351.

TURNER, B. C., C. W. TAYLOR, D. PERKINS and D. NEWMEYER, 1969 New duplication-generating inversions in *Neurospora*. Can. J. Genet. Cytol. **11**: 622–638.

VALONE, J. A., JR., M. E. CASE and N. H. GILES, 1971 Constitutive mutants in a regulatory gene exerting positive control of quinic acid catabolism in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **68**: 1555–1559.

WILLSON, C., D. PERRIN, M. COHN, F. JACOB and J. MONOD, 1964 Non-inducible mutants of the regulator gene in the "lactose" system of *Escherichia coli*. J. Mol. Biol. **8**: 582–592.

Corresponding editor: R. H. DAVIS