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Genetics

Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA

(spheroplasts/pVK88/integration/*qa* cluster)

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ABSTRACT **An efficient transformation system has been developed for *Neurospora crassa* that uses spheroplasts and pVK88 plasmid DNA. pVK88 is a recombinant *Escherichia coli* plasmid carrying the *N. crassa qa-2*$^+$ gene which encodes catabolic dehydroquinase (3-dehydroquinate hydro-lyase, EC 4.2.1.10) and is part of the *qa* gene cluster. The recipient strain carries a stable *qa-2*$^-$ mutation and an *arom-9*$^-$ mutation, thus lacking both catabolic and biosynthetic dehydroquinase activities. Transformants were selected as colonies able to grow in the absence of an aromatic amino acid supplement. These colonies were *qa-2*$^+$ and had normal levels of catabolic dehydroquinase. DNA·DNA hybridization evidence with appropriate labeled probes indicates clearly that in some instances transformation involves the integration of bacterial plasmid sequences together with the *qa-2*$^+$ gene into the *N. crassa* genome. On the basis of genetic, enzyme assay, and DNA hybridization data, at least three types of transformation events can be distinguished: (*i*) replacement of the *qa-2*$^-$ gene by the *qa-2*$^+$ gene without any effect on the expression of the other genes in the *qa* cluster, (*ii*) linked insertion of a normal *qa-2*$^+$ gene accompanied by inactivation of the adjacent *qa-4*$^+$ gene, and (*iii*) insertion of a normal *qa-2*$^+$ gene at an unlinked site in the *N. crassa* genome. This newly integrated *qa-2*$^+$ genetic material is inherited in a typical Mendelian fashion. A low level of transformation has also been obtained by using linear total *N. crassa* DNA. Two such *qa-2*$^+$ transformants are unlinked to the *qa-2*$^-$ gene of the recipient.**

Transformation has been a well-established process for the transfer of genetic material in prokaryotes for a number of years. Until recently, such a system in higher organisms (eukaryotes) remained to be firmly established. Transformation in fungi has been limited in part because of cell wall differences which restrict DNA uptake. However, a transformation system for yeast (*Saccharomyces cerevisiae*) has recently been developed through the use of enzymes that partially digest the outer cell wall (1, 2). In the initial experiments described by Hinnen *et al.* (1), a stable *leu-2*$^-$ yeast strain (a double mutant strain) was transformed with a hybrid ColE1 plasmid containing *leu-2*$^+$ DNA. Subsequently, transformation to histidine prototrophy by using a *his-3*$^+$ recombinant plasmid was also obtained (3). These transformed strains of yeast have been well characterized both for the location of the newly inserted yeast DNA and for the presence of *Escherichia coli* plasmid DNA sequences inserted into the yeast genome (1, 3).

Although presumptive evidence for transformation of inositol-requiring strains of *Neurospora crassa* has been reported (4–6), these experiments did not demonstrate the physical insertion of exogenous DNA into the *N. crassa* genome. By using a recently isolated recombinant *E. coli* plasmid (pVK88) carrying the *qa-2*$^+$ gene, which encodes catabolic dehydroquinase (3-dehydroquinate hydro-lyase, EC 4.2.1.10) of *N. crassa* (7–9), a transformation system similar to that described for yeast has been developed in *N. crassa*. The use of a recombinant plasmid as a source of a highly enriched *qa* gene and as a molecular probe provides many unique advantages, especially because of the considerable amount of genetic and biochemical information available about the *qa* gene cluster and its products. This gene cluster in linkage group VII of *N. crassa* contains four tightly linked genes, three of which encode inducible enzymes catalyzing the first three reactions in the catabolism of quinic acid: *qa-2*, catabolic dehydroquinase; *qa-3*, quinate dehydrogenase (quinate:NAD$^+$ oxidoreductase, EC 1.1.1.24); and *qa-4*, dehydroshikimate dehydratase. The fourth gene, *qa-1*, encodes a regulatory protein which in conjunction with the inducer, quinic acid, controls the expression of the three genes encoding enzymes. The gene order has been established as *qa-1*, *qa-3*, *qa-4*, *qa-2*, and the *qa* cluster is very tightly linked to the adjacent *me-7* gene (10).

pVK88 contains the *E. coli* plasmid pBR322 as well as the *N. crassa qa-2*$^+$ gene encoding catabolic dehydroquinase, an enzyme which is capable of substituting for biosynthetic dehydroquinase in either *E. coli* (7) or *N. crassa* (11). With plasmid pVK88 DNA, it has been possible to transform a stable *qa-2*$^-$ mutant (M246) to *qa-2*$^+$. The experiments demonstrate conclusively that, during certain transformation events, *E. coli* pBR322 sequences together with the *qa-2*$^+$ *N. crassa* gene on the hybrid plasmid can integrate into the *N. crassa* genome at more than one site. In addition, the *qa-2*$^+$ gene can also integrate, apparently without accompanying bacterial sequences, at more than one site. This newly integrated *qa-2*$^+$ genetic material is inherited in a typical Mendelian fashion. A low level of transformation has also been obtained by using total *N. crassa qa-2*$^+$ DNA. In this case, integration can also occur at a site (or sites) unlinked to the *qa-2* locus.

MATERIALS AND METHODS

Strains. The *N. crassa* strain (derived from wild-type 74A) used as the recipient for the transformation experiments contained a stable *qa-2* mutation (M246) combined with an *arom-9* mutation (M6-11). This double mutant lacks both biosynthetic and catabolic dehydroquinase activities and is unable to grow without a supplement of aromatic amino acids (12). In some experiments this strain carried an additional inositol-requiring mutation (89601). All presumptive transformants were crossed to the closely linked methionine-requiring strain *me-7* (4894) to obtain homokaryotic isolates and to test the linkage of presumptive transformants to the *qa-2* locus. A constitutive *qa-1*^{C} mutant (M105-R12-1.5) was used as a source of total (nonplasmid) *N. crassa* DNA (7).

DNA Preparation. pVK88 plasmid DNA was isolated from *E. coli* strain SK1572 (8) by the method of Vapnek *et al.* (13).

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Abbreviation: kb, kilobase.

Linear *N. crassa* DNA (having an average molecular weight of 26×10^6) for use in transformation experiments was isolated from *qa-1*^{C} mutant 105 by the method of Hautala *et al.* (14). DNA for Southern gels (15) was extracted from transformants by the following procedure. One gram of coarsely ground mycelia was mixed with 20 ml of 10 mM Tris·HCl/1 mM EDTA/4% sodium dodecyl sulfate, pH 8, and then an equal volume of phenol/chloroform/isoamyl alcohol, 49:49:2 (vol/vol), was added. The supernatant was treated for 1 hr at room temperature with proteinase K (Beckman) at 0.5 mg/ml. Nucleic acids were precipitated by ethanol at −20°C. The DNA was further purified by digestion for 1 hr at 37°C with preheated RNase (bovine pancreas, type II, Sigma) at 10 μg/ml, reprecipitated with ethanol at −20°C, and finally dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 8. Proteins were removed with the phenol/chloroform/isoamyl alcohol mixture after the addition of proteinase K and RNase.

Transformation of *N. crassa*. The initial transformation experiments, including spheroplast formation, followed the general procedures outlined for transformation in yeast by Hinnen *et al.* (1). Some modifications were made in this procedure. A conidial suspension (1×10^7 conidia per ml in 150 ml of Fries minimal sucrose supplemented with aromatic amino acids) was germinated for 4 hr at 25°C, washed thoroughly with distilled water, and concentrated by centrifugation. The pellet was resuspended in 10 ml of 1 M sorbitol (Sigma), and the suspension was treated with 3% gluculase (Endo Laboratories, New York) for 1 hr at 30°C. The spheroplasts were washed three times with 20 ml of 1 M sorbitol and were resuspended in 0.5 ml of 1 M sorbitol/10 mM 4-morpholinepropanesulfonic acid (Sigma)/50 mM $CaCl_2$, pH 6.3, containing 5 μl of dimethyl sulfoxide. This suspension was heated for 30 sec at 37°C and then placed on ice. Plasmid DNA was then added to a final concentration of 60 μg/ml. After cooling on ice for 30 min, the suspension was heated for 30 sec at 45°C and 5 ml of 40% polyethylene glycol 4000 (Sigma)/10 mM 4-morpholinepropanesulfonic acid/50 mM $CaCl_2$, pH 6.3, was added. After 20 min at room temperature the spheroplasts were centrifuged and resuspended in 8 ml of the pH 6.3 sorbitol buffer. Aliquots (1 ml) were added to 50 ml of regeneration agar (1 M sorbitol/3% agar/sorbose-fructose-glucose Fries minimal medium) and overlayered onto four minimal sorbose-fructose-glucose 1.5% agar plates. Plates were incubated at 30°C. The frequency of spheroplast formation was determined by plating at an appropriate dilution into both sorbose-fructose-glucose 1.5% agar containing a supplement of aromatic amino acids and into regeneration agar which was then overlayered onto an aromatic amino acid supplemented plate.

Hybridization Analysis of Restriction Endonuclease Digests. Total *N. crassa* DNA was digested with *Xho* I (New England BioLabs). The products were separated on 0.8% agarose gels and transferred to nitrocellulose filters (Millipore HAWP00010) by the method of Southern (15). Details of these procedures and the hybridization conditions have been described (8). ^{32}P-Labeled plasmid DNA was prepared by nick translation with *E. coli* DNA polymerase I (Boehringer Mannheim) (16).

Genetics Techniques and Enzyme Assays. Plating and crossing techniques, media composition, and procedures for the induction and assay of the three *qa* enzymes have been described (17, 18).

Containment Considerations. These experiments were performed under P2 laboratory conditions as approved by the local Biosafety Committee and by the Recombinant Advisory Committee of the National Institutes of Health.

RESULTS

DNA-Mediated Transformation of the *qa*-2 Gene. Spheroplasts of the recipient strain were mixed with pVK88 plasmid DNA or linear *Neurospora* total DNA (from *qa-1*^{C} mutant 105) in the presence of $CaCl_2$, sorbitol, morpholinepropanesulfonic acid, and dimethyl sulfoxide and subjected to the treatments described in *Materials and Methods*. Various buffers, various pHs and various $CaCl_2$ concentrations were tried. The most significant feature in increasing transformation in these experiments appears to be the temperature shocks. Under the optimal conditions described in *Materials and Methods*, transformants have been obtained at low frequency (≈0.1/μg of DNA) with linear total DNA from *qa-1*^{C} mutant 105. The frequency of transformation increased as much as 300-fold, to 5–30 transformants per μg of DNA, with the use of pVK88 plasmid DNA. Colonies begin to appear in 3 days at 30°C. *qa-2*$^+$ colonies were obtained only after addition of either total *N. crassa* DNA or pVK88 plasmid DNA during the transformation experiments. Most of these colonies were able to grow on quinic acid as a carbon source but still retained the *arom-9* mutation as demonstrated by enzyme assays indicating the presence of catabolic dehydroquinase activity due to the accumulation of dehydroquinic acid (12). Occasional colonies found on control plates and a few among the many transformants isolated from DNA-treated conidia were reversions of the *arom-9* mutant which is known to revert spontaneously at a frequency of $<1 \times 10^{-9}$. No spontaneous reversions of *qa-2* allele M246 have been detected in $>1.5 \times 10^{10}$ macroconidia. Previous experiments also indicate that this allele does not revert after ultraviolet light treatment (18).

Classification of Transformants Obtained with Hybrid Plasmid DNA. Tetrad analyses of crosses of DNA-induced *qa-2*$^+$ transformants indicate that the inheritance of the qa-2$^+$ phenotype is Mendelian, not cytoplasmic, and that transformants are usually isolated as heterokaryons. At the present time, >1000 presumptive transformants have been obtained by using pVK88 plasmid DNA. However, only a limited number have as yet been characterized genetically and by *qa* enzyme assays. Three types of transformants have been observed in crosses to the *me-7* strain from a sample of 14 independently occuring transformants.

Replacement Type. In this type (6 of 14), the resulting qa-2$^+$ phenotype is closely linked to the *me-7* gene and hence to the *qa* region. The transformants have essentially normal activities for the three *qa* enzymes. Representative data from a cross of this type (TN1-5-1) to the *me-7*$^-$ strain are given in Table 1. All 17 complete tetrads segregated 4:0 for the *qa-2*$^+$ gene and 2:2 for the *me-7*$^-$ allele.

Linked Insertion Type. The qa-2$^+$ phenotype of this type (2 of 14) is also closely linked to *me-7*. The transformants have normal levels of the *qa* enzymes catabolic dehydroquinase (*qa-2*) and quinate dehydrogenase (*qa-3*) but lack dehydroshikimate dehydratase activity (*qa-4*). The original transformants of this type are able to grow without an aromatic supplement because they are qa-2$^+$ and produce catabolic dehydroquinase by internal induction [since they are *arom-9* mutants and accumulate dehydroshikimate (11) but cannot grow on quinic acid because they lack dehydroshikimate dehydratase activity]. Representative tetrad data for a cross of TN1-48 to the *me-7*$^-$ strain are given in Table 1. All 11 complete tetrads segregated 4:0 with respect to the *qa-2*$^+$ gene and 2:2 with respect to the absence of dehydroshikimate dehydratase activity. All of the latter isolates were *me-7*$^+$, indicating close linkage to the *me-7* gene of the transformed *qa-2*$^+$ gene and of the newly appearing qa-4$^-$ phenotype.

Unlinked Duplication Type. In the duplication type (6 of

Table 1. Tetrad data from three types of transformants detected to date.

Acrospore number	Gene segregations			Specific activities of *qa* enzymes*		
	qa-2	*me-7*	*arom-9*	QDHase	C-DHQase	DHS-Dase
Replacement type (TN1 5-1)						
3.1	+	+	−	46	61	31
3.4	+	+	+	61	92	26
3.5	+	−	−	44	66	16
3.7	+	−	+	25	56	17
Linked insertion type (TN1-48)						
4.1	+	−	−	15	38	18
4.4	+	−	−	17	34	12
4.5	+	+	+	24	40	0
4.7	+	+	+	19	35	0
Unlinked duplication type (TN5-2)						
2.1	+	+	−	27	35	42
2.4	−	+	+	77	0	39
2.5	+	−	−	31	33	29
2.7	+	−	+	44	52	23

Gene segregations and levels of *qa* enzyme activities are indicated for a representative tetrad of each type. The tetrads were isolated from a cross to *me-7*$^-$ of transformants induced in the recipient strain *qa2* (M246) *arom-9* (M6-11) by pKV88 plasmid DNA.

* Mycelia were grown on 1.5% sucrose in shake flasks for 24 hr and then shifted for 6 hr to medium containing 0.1% quinic acid (17). QDHase, quinate dehydrogenase; C-DHQase, catabolic dehydroquinase; DHS-Dase, dehydroshikimate dehydratase.

14) the *qa-2*$^+$ gene is unlinked (or very loosely linked) to the original *qa-2* locus and to *me-7*, and the transformants have normal activities for the *qa* enzymes. Representative tetrad data are given in Table 1. In this example, the tetrad exhibits a 3:1 segregation for *qa-2*$^+$:*qa-2*$^-$. In crosses involving this transformant (TN5-2), 26 complete tetrads examined gave the following segregation data (*qa-2*$^+$:*qa-2*$^-$): 8 were 2:2; 3 were 4:0; and 15 were 3:1. All *qa-2*$^-$ segregants were also *me-7*$^+$. Thus, the transformant has the *qa-2*$^+$ gene inserted at another site not closely linked to the *me-7* gene and at a considerable distance from the centromere (≈29 map units based on the frequency of second division segregations). A subsequent cross to the *me-7*$^-$ strain of the isolate from spore 2.1 (which is phenotypically qa-2$^+$) indicated the presence of the other *qa-2*$^-$ allele in this isolate, as expected on the basis of its close linkage with *me-7*$^+$. Segregation data from a cross between the isolates from spores 2.4 and 2.7 were in agreement with the expectation that the transformed strain carried two *qa-2* genes, with the newly inserted *qa-2*$^+$ gene at a site not linked to the original *qa-2*$^-$ locus.

Mitotic Stability of Transformed Strains. Conidia from homokaryotic isolates obtained from crosses of transformed strains to the *me-7*$^-$ strain were plated to detect the possible presence of the original *qa-2*$^-$ genotype as an indication of mitotic instability. In limited platings to date of transformed strains from each of the three genetic types obtained with hybrid plasmid DNA, no *qa-2*$^-$ isolates have been detected. Thus, there is as yet no evidence for mitotic instability in *N. crassa* transformants as has been noted for type I transformants in yeast (1).

Transformants Obtained with Linear Total *N. crassa* DNA. At the present time, only two of four *qa-2*$^+$ transformants obtained by using total DNA from the *N. crassa qa-1*^{C} mutant 105 have been characterized genetically. Both of these transformants are of the unlinked duplication type with the newly inserted *qa-2*$^+$ genes located at the same or different sites unlinked to the *qa-2*$^-$ locus.

DNA Hybridization Results. In order to determine if pBR322 bacterial DNA sequences or additional *N. crassa* DNA sequences were inserted into the *N. crassa* genome during transformation, hybridization experiments were performed with DNA isolated from *N. crassa* transformants. In these experiments, two ^{32}P-labeled DNA probes were used: pVK88 plasmid DNA containing both pBR322 and *N. crassa qa-2*$^+$ sequences, and pBR322 plasmid DNA alone. Total DNA extracted from both the parental recipient strain (M246, *arom-9*) and from 12 independently isolated transformants (5 replacement types, 2 linked insertion types, and 5 unlinked duplication types) was digested with *Xho* I. After electrophoresis on 0.8% agarose, the fragments were hybridized to either ^{32}P-labeled pVK88 (Fig. 1) or pBR322 (Fig. 2) plasmid DNA. Hybridization of DNA from the parental strain (M246, *arom-9*) yielded two fragments because the 6.8-kb *N. crassa* fragment in pVK88 contains a single *Xho* I site (unpublished results). (The additional larger fragment in Fig. 1, lane F, resulted from partial digestion of the *N. crassa* DNA.) As expected, no hybridization with pBR322 was observed (Fig. 2, lane F).

Neither of the replacement type transformants shown (isolates TN1-5-1 and TN13-3-1.1) contained pBR322 sequences and both gave gel patterns indistinguishable from the control when hybridized with pVK88 DNA. In the case of the two linked insertion types, one isolate from TN1-48 and two independent isolates from TN6-2-7 (1.7 and 4.1) were used in the DNA hybridization studies. The two isolates of TN6-2-7 were obtained from two different asci from a cross of the original transformant to the *me-7*$^-$ strain. All three isolates of the linked insertion type exhibited altered patterns of hybridization with pVK88. One isolate (TN6-2-7-4.1) also contained pBR322 sequences; the other two isolates (TN1-48-4.5 and TN6-2-7-1.7) did not. Of the two unlinked duplication types shown, TN5-2-2.1 had no bacterial sequences and TN13-4-5.5 did; both

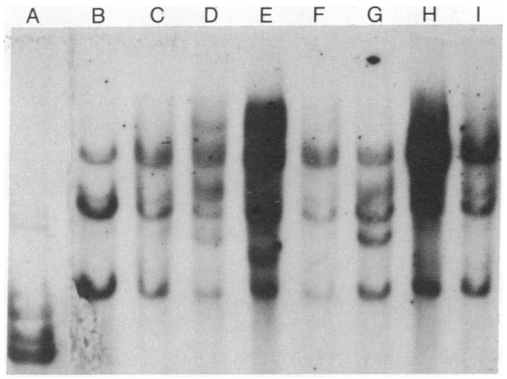

FIG. 1. DNA·DNA hybridization of ^{32}P-labeled pVK88 plasmid DNA to *N. crassa* DNA. DNA (20–50 μg) from each of eight *N. crassa* strains was cleaved with *Xho* I, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose paper. The DNA was then hybridized with ^{32}P-labeled pVK88 DNA. Lanes contain DNA from the following sources: A, SK1572 (pVK88) digested with *Pst* I and *Xho* I; B and C, two replacement type transformants, TN1-5-1 (B) and TN13-3-1.1 (C); D and E, two unlinked duplication type transformants, TN5-2-2.1; (D) and TN13-4-5.5 (E); F, recipient strain M246; G, H, and I, two linked insertion type transformants, TN1-48-4.5 (G), and TN6-2-7, isolates 4.1 (H) and 1.7 (I). *Xho* I cleaves the 6.8-kilobase (kb) *Pst N. crassa* fragment contained in pVK88, generating two fragments (the lowest two bands visible in A). The next band above represents intact pBR322 (4.3 kb). The nontransformed *N. crassa* control M246 (lane F) showed three hybridization bands in this experiment. The lower two (4.5 and 6.6 kb) represent hybridization to the two *Xho* I *N. crassa* fragments contained within pVK88. The band with the slowest mobility represents a partial digestion product of these two fragments. Lanes B, C, and I gave patterns similar to lane F, the other isolates yielded strikingly different patterns (see text). All samples are run on the same gel, but the pVK88 had to be exposed for a shorter period of time.

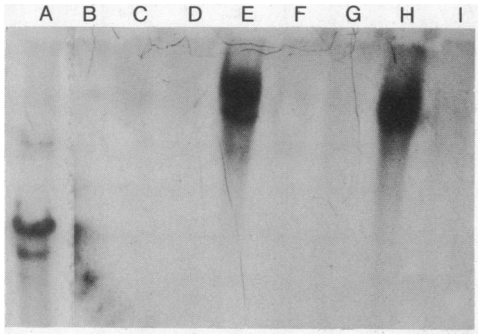

FIG. 2. DNA·DNA hybridization of ^{32}P-labeled pBR322 plasmid DNA to *N. crassa* DNA. DNA (20–50 μg) from each of eight *N. crassa* strains was cleaved with *Xho* I, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose paper. The DNA was then hybridized with ^{32}P-labeled pBR322 DNA. Lanes contain DNA as follows: A, SK1571 (pBR322) digested with *Pst* I; B, transformant TN1-5-1; C, transformant TN13-3-1.1; D, transformant TN5-2-2.1; E, transformant TN13-4-5.5; F, recipient strain M246; G, transformant TN1-48-4.5; H, transformant TN6-2-7-4.1; I, transformant TN6-2-7-1.7. The intense band in lane A represents unit-length pBR322 (4.3 kb).

exhibited additional hybridization bands corroborating the genetic data showing lack of linkage to *qa-2*$^-$. Of the 12 transformants tested, 3 contained pBR322 associated with *N. crassa* DNA sequences—2 unlinked duplication types (Fig. 2 and other data) and one linked insertion type (Fig. 2, lane H).

DISCUSSION

The experiments described in this paper provide clear evidence for the occurrence of transformation in *N. crassa*. The production of spheroplasts is an essential factor in transformation because the omission of the glusulase treatment during an otherwise typical transformation experiment results in the absence of transformants. The frequency of spheroplast formation varies, ranging from 50 to 85% of the treated conidia and apparently depending upon the age and batch of glusulase. In contrast to yeast, in *N. crassa* there is essentially no loss in viability after spheroplast formation and regeneration. Thus, ≈100% of the conidia and spheroplasts in a glusulase-treated conidial suspension give rise to colonies. The transformation frequency does not seem to be totally dependent on the frequency of spheroplast formation, and other factors are undoubtedly involved in DNA uptake.

The results of the DNA hybridization analysis, combined with the genetic and enzyme assay data, indicate that transformation in *N. crassa* involves at least three different types of events. In the replacement type, the original *qa-2*$^-$ allele in the recipient strain has apparently been replaced by the *qa-2*$^+$ gene on pVK88 without the introduction of adjacent bacterial sequences. The resulting transformed strains have normal levels of catabolic dehydroquinase activity and of the other two *qa* enzyme activities. This type can be considered equivalent to the type III transformant observed in yeast (1). The simplest hypothesis to explain this type of replacement would appear to involve the occurrence of a double crossover within paired homologous *qa* or closely adjacent *N. crassa* DNA sequences of the recipient strain and of pVK88 plasmid DNA.

In the linked insertion type, the data suggest that in transformant TN1-48-4.5 (Table 1; Fig. 2, lane G) a fully functional *qa-2*$^+$ gene from pVK88 has been inserted without the introduction of any adjacent bacterial sequences. However, of particular interest is the fact that this transformant lacked detectable dehydroshikimate dehydratase activity. Genetic evidence (19) has established the order of the four genes in the *qa* cluster as *qa-1*, *qa-3*, *qa-4*, *qa-2*. Thus, the *qa-4* gene which encodes dehydroshikimate dehydratase is adjacent to the *qa-2* gene which encodes catabolic dehydroquinase. Consequently, in this instance transformation does not involve a simple replacement of the *qa-2*$^-$ gene by a *qa-2*$^+$ gene. Although the DNA hybridization data from isolate TN1-48-4.5 indicate the absence of bacterial sequences, a pattern distinct from that of the M246 control was observed (Fig. 1, lane F). This observation suggests that there is a difference in the organization of the DNA in the vicinity of the *qa-2* gene and is compatible with some type of abnormal insertion of *N. crassa* DNA from pVK88 (presumably into the *qa-4*$^+$ gene) during transformation which restores normal *qa-2*$^+$ function but leads to the loss of function for the adjacent *qa-4*$^+$ gene. This effect must be relatively localized, however, because it does not modify expression of the *qa-3*$^+$ gene which is on the opposite side of the *qa-4*$^+$ gene. The possibility remains that transformants in this category may be comparable to the type I transformants of yeast (1) such that, after crossing, the bacterial sequences initially present are retained in some isolates (e.g., TN6-2-7-4.1) and are lost in other isolates (e.g., TN6-2-7-1.7).

In the unlinked duplication type, the newly inserted *qa-2*$^+$ gene is located on a linkage group other than VII, the location of the *qa-2*$^-$ gene in the recipient. This type of transformant has normal levels of all three *qa* enzymes. In certain instances (e.g., TN13-4-5.5) the presence of bacterial sequences has been demonstrated (Fig. 2, lane E). In others, however (e.g., TN5-2-2.1), no such sequences have been detected (Fig. 2, lane D). This category is generally comparable to the type II category in yeast (1) and suggests that there are *N. crassa* DNA sequences located in the *qa* region carried on pVK88 that are reiterated in the genome and permit insertion at more than one site. In this respect, the *qa* region in *N. crassa* is comparable to the *leu-2* region and differs from the *his-3* region in yeast (3). DNA hybridization analysis (Fig. 1, lanes C and D) suggests that the unlinked duplication types involve integration at different sites, as has been demonstrated in yeast (3). The limited data available suggest that *N. crassa* and yeast may differ in the frequency with which bacterial sequences are integrated along with eukaryotic DNA in unlinked duplication types. The apparent lower frequency in *N. crassa* may indicate that double crossover events occur more often within the eukaryotic DNA carried by pVK88 than within that carried by pY*eleu10*. However, the possibility of initial integration of the bacterial sequences by a single Campbell-like recombination event (20), followed by more frequent subsequent loss in *N. crassa*, cannot be rigorously excluded.

The ability to obtain transformation with linear total *N. crassa* DNA indicates clearly that the bacterial sequences present on pVK88 are not required for integration. The data also demonstrate that the use of plasmid DNA greatly increases the frequency of transformation, as would be expected because the DNA is greatly enriched for the *qa-2*$^+$ gene. Of particular interest is the evidence that integration of the linear nonplasmid DNA can occur at a site (or sites) unlinked to the *qa-2* gene. This result provides further support for the conclusion that repetitive DNA sequences exist adjacent to the *qa* gene cluster. Finally, it seems highly probable that integration of the linear *qa* DNA involves a double crossover mechanism such as has been postulated to explain the replacement type transformation event.

As in the case of yeast, the development of an efficient transformation system for *N. crassa* greatly expands the possibilities of manipulating this organism genetically. This is especially true because the DNA from the *qa* gene cluster uti-

lized in these initial experiments has been found to integrate at more than one site in the *N. crassa* genome. This *qa* DNA with its associated bacterial sequences should make possible many novel types of transformation experiments and lead to a better understanding of *N. crassa* gene organization and function, not only in the *qa* gene cluster but also in the *N. crassa* genome in general.

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