Some Property of the Nucleus Determines the Competence of *Neurospora crassa* for Transformation

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ABSTRACT

In Neurospora, transformation of spheroplasts is quite efficient and usually occurs with the transforming DNA integrated at ectopic sites in the chromosome. However, only a small fraction of the spheroplasts is actually competent for transformation. To distinguish whether the limitation to competence is at the level of the plasma membrane or at the level of the nucleus, we performed experiments in which heterocaryotic spheroplasts were required to integrate two different plasmids in one transformation procedure. The cotransformants were then analyzed to determine into which nucleus or nuclei the separate plasmids had integrated. Results of such experiments confirm that successful ectopic transformation in *Neurospora crassa* requires a competent nucleus. The integration patterns of the two separate plasmids indicate that the availability of appropriate chromosomal sites for ectopic integration may be an aspect of nuclear competence.

STABLE transformation of a eucaryotic cell by exogenous DNA requires the breaching of at least two potential barriers. The DNA must somehow end up inside both the plasma membrane and the nuclear membrane. In higher eucaryotes, the nuclear membrane disappears during mitosis. Therefore it is possible, at least in principle, for transforming DNA to become part of the nuclear genome during the time the nuclear membrane is absent. In filamentous fungi and in yeast, however, the nuclear membrane remains intact throughout mitosis, so that it cannot be dismissed *a priori* as a barrier to the transforming DNA.

The frequency of transformation of yeasts and filamentous fungal cells having intact cell walls is usually vanishingly low. Therefore, the experimenter usually converts the cells to spheroplasts before attempting to transform them. However, even among spheroplasts, only a small minority of the cells exposed to exogenous DNA undergoes transformation. This could be explained by supposing that all target cells are functionally identical, but that transformation is a rare, random event. Conversely, it can be supposed that even a clonally derived population of cells becomes subtly heterogeneous, so that a small minority of the cells are "competent" to be transformed and the rest are not. In a representative experiment to test these models, a double auxotroph, which we can designate A^-B^-, may be singly transformed to A^+B^- at a low frequency by plasmid DNA carrying A^+, and it may be singly transformed at a similar frequency to A^-B^+ by plasmid DNA carrying B^+. What might happen if A^-B^- target cells were simultaneously exposed to a mixture of the two plasmids? The "identical targets" model would predict that cotransformation would be very rare indeed, its frequency being the product of two frequencies that are themselves quite low. The "competent cells" model predicts that many or most cells that are transformed by one kind of plasmid would be transformed by the other as well. The evidence heavily favors the "competent cells" model, (AUSTIN and TYLER 1990), though competence may be a relative condition encompassing a range of susceptibilities to transformation.

If only certain cells are competent, we may ask what keeps the rest of the cells incompetent. On one hand, it is possible that a small number of the cells have plasma membranes that are permeable to the transforming DNA and this limits frequency of transformation. On the other hand, while DNA must get into the cell, competence may be limited by some other factor such as the characteristics of the nuclei present in that cell. We wanted to determine which of these was the limiting factor.

Consider target spheroplasts that are coenocytic and that all nuclei are A^-B^-, but that the cells are heterocaryotic with respect to some other markers—that is, the nuclei are $A^-B^-Y^-Z^+$ and $A^-B^-Y^+Z^-$. Consider the possible outcomes if these target cells are then cotransformed with a mixture of plasmids carrying A^+ and B^+ to give a prototrophic heterocaryon. If the plasma membrane is a much more important limitation to competence and all nuclei are equal with respect to competence, then the minority of cells with a highly permeable plasma membrane will often admit two or more plasmids and these will distribute themselves randomly between the two kinds of nuclei (see Figure 1A). If a

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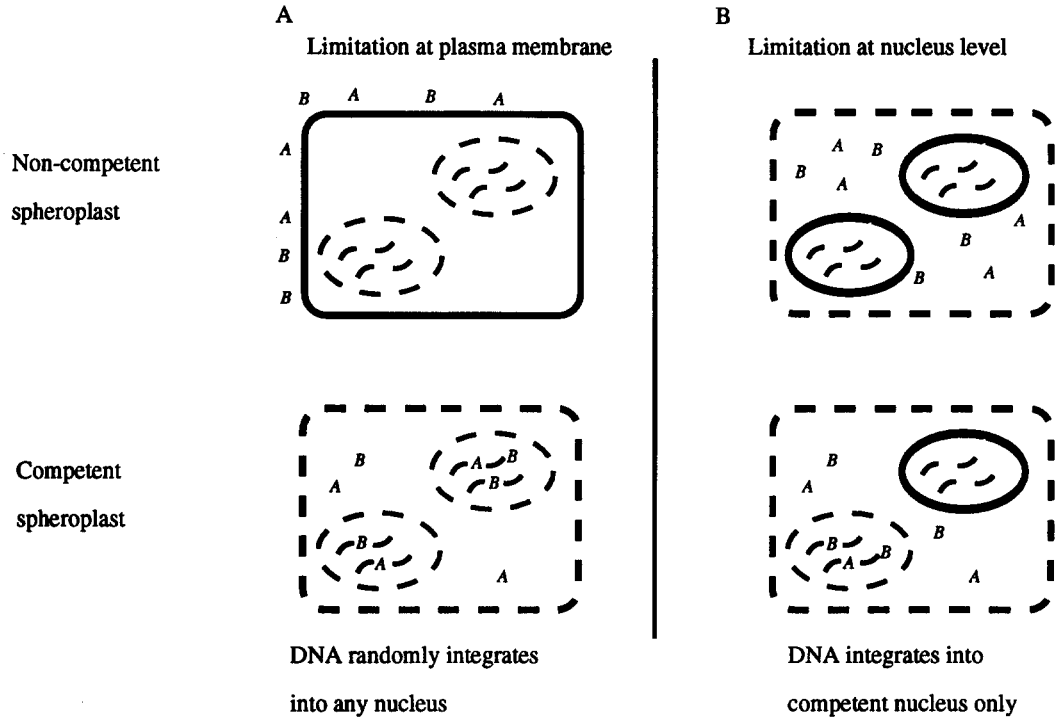

FIGURE 1.—Cartoon representation of a heterocaryotic cell (spheroplast) and two possible frequency-limiting barriers to competence for transformation (A and B). Solid lines represent frequency-limiting barriers to the plasmid DNAs (*A* and *B*) and the presence (top) or absence (bottom) of these blocks is what determines competence.

number of such cotransformed heterocaryons are then resolved to their constituent homocaryons, one would expect to find a random distribution of transformation events as suggested in Table 1.

In contrast, it is possible that the major limitation to competence is determined by the presence or absence of a competent nucleus. In such a case, we can visualize the cytoplasm of many or all spheroplasts containing A^+ and B^+ plasmids, but the plasmids only enter and are successfully integrated in a rare (competent) nucleus (see Figure 1B). If this is a correct picture, one would expect that transformation would usually be limited to one nucleus, so that resolution of any particular heterocaryotic transformant would yield mainly class I type transformants (see Table 1). Previous studies indicate that when a multinucleate protoplast is transformed the transforming DNA usually integrates into only one of the nuclei present (PANDIT and RUSSO 1992) but other interpretations are possible. Our results confirm and extend their suggestion that only an occasional nucleus is competent for transformation.

MATERIALS AND METHODS

Media: For production of mycelia and conidia, strains were grown in 1 ml of Vogel's Medium N containing 2% sucrose, supplemented as indicated in the individual experiments, or in 20- to 50-ml batches solidified with 1.5% agar. Spheroplasts were allowed to regenerate their cell walls in "liquid regeneration medium," which contained Vogel's salts (1×), sucrose (1%), glucose (1%), $MgSO_4$ (0.5 M), and, because of the am_{132} mutation in these strains, leucine (5 mM). All platings were done on Vogel's salts with the sugar mixture of BROCKMAN and DESERRES (1963) to induce colonial growth, solidified with 1.5% agar and supplemented as described in the individual experiments.

TABLE 1

Classes of possible transformation genotypes

Class	Description	No. of possible combinations within class
I	One nuclear type transformed with both plasmids One nuclear type untransformed	2
II	One nuclear type transformed with one plasmid One nuclear type transformed with other plasmid	2
III	One nuclear type transformed with both plasmids One nuclear type transformed with one of the plasmids	4
IV	Both nuclei transformed with both plasmids	1

Strains and plasmids: The usual host for transformation in this study was a forced heterocaryon (RLM 56–40) constructed from strains *pan-2; cyh-1; qa-2; aro-9; inl; am$_{132}$a* (RLM 55–18) and *nic-3; mep^r; cot-1; qa-2; aro-9; inl; am$_{132}$a* (RLM 56–11). The heterocaryotic strain was isolated by selecting for growth on medium containing aromatic amino acids, inositol and leucine as the only supplements. The *aro*-9 allele was included in the strains to allow for selection of *qa-2$^+$*. An *aro*-9, *qa*-2 double mutant requires aromatic amino acids for growth, though each single mutant is prototrophic (PERKINS *et al.* 1982). (The *am$_{132}$* mutation was included in both nuclear types so that a third target for transformation would be available if needed; as the experiment actually developed, it was not needed.) Plasmid pRAL-1, which contains the *N. crassa qa-2$^+$* gene (AKINS and LAMBOWITZ 1985), can transform the heterocaryon to independence for aromatic amino acids. Plasmid pOKE01 is a pUC18 vector containing a 8.5-kbp *Pst*I fragment from pRAL724593 (AKINS and LAMBOWITZ 1985), that is able to complement the *inl* mutation.

Spheroplast preparations and transformations: Competent spheroplasts of the heterocaryotic host strain were prepared according to the method of SCHWEIZER *et al.* (1981), using the modifications of AKINS and LAMBOWITZ (1985). The spheroplasts were exposed to equimolar amounts of pRAL-1 and pOKE01 and were then incubated in liquid regeneration medium at 25° with gentle agitation for 12–16 hr and the resulting walled cells were plated. Strains that were cotransformants for the *qa-2$^+$* and *inl$^+$* genes were selected on Vogel's minimal plates supplemented only with leucine (5 mM). In certain controls, cells subjected to both plasmids were put under selection only for *qa-2$^+$* or *inl$^+$* by supplementing the medium appropriately. When a strain specifically transformed for only one of the genes was desired, the cells were transformed with the corresponding plasmid alone. Heterocaryons synthesized by mixing singly transformed strains were converted to spheroplasts and subjected to a mock transformation procedure in which DNA was omitted from the transformation mixture.

Resolution of heterocaryons to homocaryons: The transformants of the heterocaryons were picked to tubes of liquid selective medium (Vogel's minimal plus leucine) and allowed to conidiate. Conidia from these tubes were streaked to selective plates. Well-separated colonies were transferred to tubes of Vogel's minimal plus leucine medium and allowed to conidiate. To resolve these heterocaryotic isolates into the homocaryons representing the nuclear types of which they were composed, we streaked conidia of each isolate to medium permissive for growth of one of the nuclear types without selection for either of the transformation markers. To select for the homocaryon derived from the *pan-2* nucleus, we streaked the conidia to medium containing pantothenic acid, inositol, aromatic amino acids and cycloheximide. Because *cyh-1* is semirecessive, only those cells that are homocaryotic for it should grow vigorously. Therefore this medium allows growth of homocaryons that are *pan-2; cyh-1; qa-2; aro-9; inl.* Five colonies representing the *pan-2* nucleus from each heterocaryon were picked, and the requirement for pantothenic acid was confirmed. To select for the derivative of the *nic-3* nucleus, we streaked the conidia to medium containing nicotinamide, inositol, aromatic amino acids and 6-methylpurine. Isolates that grow on these plates are expected to be homocaryotic because *mep^r* is recessive; they are expected to require nicotinamide, but they may or may not require the other added supplements. These plates were also incubated at 34°, a temperature at which cells that are *cot-1* homocaryons display a characteristic colonial morphology. Five *cot-1* colonies representing the *nic-3* nucleus from each original heterocaryon were picked and the requirement for nicotinamide was confirmed. All tubes were incubated at 30° and the isolates were allowed to conidiate.

Determination of transformation phenotype of homocaryotic isolates: Conidial suspensions were made from each homocaryotic isolate and were spotted to plates to determine whether the isolates required inositol and/or aromatic amino acids.

DNA preparations and restriction digests: Neurospora genomic DNA for Southern blots was prepared by a modification of the method of METZENBERG and STEVENS (1982) and digested with *Cla*I (Promega). Intact Neurospora chromosomes were prepared and digested with *Not*I (Promega) according to the method described by BUTLER and METZENBERG (1989).

Electrophoresis and hybridizations: CHEF gel electrophoresis was performed with the apparatus described by CHU *et al.* (1986). Gels were run according to BUTLER and METZENBERG (1989) with the exception that the gel was a step gradient 0.7%-1.3% agarose gel. Southern blotting and hybridizations were performed with Zetabind membrane as recommended by the manufacturer (AMF CUNO, Meriden, CT). Probes used were an 8.5-kbp *Pst*I fragment of the *inl* gene from pOKE01 and an 0.8-kbp *Hpa*I-*Eco*RI fragment of the *qa-2* gene from pRAL-1.

Recovery of nonintegrated DNA from *N. crassa* cells post-transformation: The host cells were exposed to the two plasmids as usual and the mixture was divided into three equal portions. Two of the portions were added to regeneration medium and allowed to incubate at room temperature overnight with gentle agitation. The cells from the third portion were collected by centrifugation. They were then suspended in STE buffer (1 M sorbitol, 100 mM Tris, pH 8.0, 50 mM EDTA). The cells were lysed by extraction with phenol/chloroform (1:1, saturated with 100 mM Tris, pH 8.0, 50 mM EDTA). After two more extractions, the DNA was precipitated with ethanol, washed, and dissolved in TE (10 mM Tris, pH 8.0, 1 mM EDTA).

DNA was recovered from regenerated cells as follows. The cells were harvested by centrifugation and then suspended in lysing enzymes (2 mg/ml) in 1 M sorbitol, 50 mM EDTA, pH 8.0. After 1 hr. at 30° with shaking at 100 RPM, the cells were harvested by centrifugation and then suspended in STE buffer. The solution was adjusted to 1% SDS, 0.5 mg/ml proteinase K, and incubated at 50° for 15 min. This was followed by three extractions with an equal volume of phenol/chloroform. The DNA was precipitated with ethanol, washed, and dissolved in TE.

The recovered DNA was examined for the presence of any coligated plasmids by the following methods. A portion of the DNA was analyzed directly by Southern analysis. Another portion of the DNA was used to transform competent DH5α bacterial cells. We selected for cells resistant to ampicillin (a marker present on the plasmid pOKE01), cells resistant to chloramphenicol (a marker present on the pRAL1 plasmid) and cells resistant to both ampicillin and chloramphenicol. Plasmid DNA recovered from resistant colonies was digested with the restriction enzyme *Hpa*I and size-fractionated by electrophoresis through a 1% agarose gel.

RESULTS AND DISCUSSION

A competent nucleus is necessary: To examine what determines the competence of a spheroplast, we took advantage of the ability of *N. crassa* to grow as a heterocaryon. We prepared a strain, which, when grown under selective conditions, was forced to carry at least one representative each of two nuclear types in a common

cytoplasm. Spheroplasts of this strain were cotransformed to independence for aromatic amino acids and inositol by incorporation of the two plasmids: pRAL-1 and pOKE01, respectively. The cotransformed, heterocaryotic isolates were then resolved into their homocaryotic components and the dependence or independence of the component of each nuclear type with respect to inositol and to aromatic amino acids was determined (see Figure 2).

We analyzed a total of 52 random, heterocaryotic, cotransformed isolates from three independent transformation experiments. The results are shown in Table 2. All of the isolates contained at least one homocaryotic component that was cotransformed to independence for inositol and aromatic amino acids. The majority of them (49) had only one component (nuclear type) that displayed any type of transformation event at all. Growth under our selection conditions means that both plasmid DNAs had to be present in a cytoplasm that was shared by at least two nuclei. If all of the nuclei present were open to transformation, more multiple-nuclear transformation events should have been observed. Thus it appears that not all *nuclei* present in a common, "competent" cytoplasm are competent. In most cases it is a single nucleus that has the necessary characteristics for successful transformation. Our conclusion is thus similar to that of PANDIT and RUSSO (1992), but the nature of our evidence is quite different. Those authors transformed a heterocaryon and selected for expression of a single plasmid-borne gene encoding hygromycin resistance. The design of their experiment was such that no spheroplast needed to be more than singly transformed to be detected under selective conditions. If one nucleus had incorporated a hygromycin resistance plasmid, there would be little or no selective pressure to maintain a second nucleus which had also incorporated such a plasmid. In our work, retention of both plasmids was kept under selective pressure until the heterocaryons were deliberately resolved, so there was no risk of a second transformed nucleus going undetected.

It seemed possible that a strain transformed singly in each nuclear type might be unable to grow as a heterocaryon. If this were so, our experimental procedure would have selected against those types of transformants. To check whether such heterocaryons would be able to grow, we transformed spheroplasts of the homocaryotic components with either pOKE01, pRALI or both plasmids to obtain homocaryotic cultures with all possible transformation phenotypes. We then combined conidia of these to prepare each of the four possible class I and II type heterocaryons (Table 1). The resulting heterocaryons were examined for any growth differences under selective conditions identical to those used for the previous experiment, both conidia and spheroplasts were examined. All isolates grew well and indistinguishably, indicating that the two plasmids can co-complement even if they reside in separate nuclei. Thus the ability of the cotransformants to grow would not have been dependent on the distribution of the transformation events between the two kinds of nuclei.

It remains possible that the actual selection for *expression* of *both* plasmids was a determining factor as to the type of results we observed. To test this, we exposed the heterocaryotic spheroplasts to both plasmids simultaneously but selected for only one of them. The resulting transformants were then resolved into their respective homocaryotic components and the distribution of transformation events between the two kinds of nuclei was determined. In each case, we found that only one of the components of each particular heterocaryon was transformed for the selected plasmid. Of those transformed by the selected plasmid, 75% were also transformed by the unselected plasmid. These results indicate that selecting for expression of *both* plasmids in our initial cotransformation experiment did not select against any of the hypothetically possible types of transformation events. The results from the above experiments confirm that competency of the plasma membrane to take up DNA is not sufficient to make a spheroplast competent to be transformed. Characteristics of the nucleus are important in the success of a transformation event. It is known that nuclei in a common dormant conidium are arrested at various stages in the cell cycle (SERNA and STADLER 1978). It is reasonable to think that nuclei in a common protoplast divide asynchronously and that there is a narrow window of competence during the nuclear cycle which accounts for the occasional, competent nucleus.

Localization of integrated DNA: To confirm the molecular events inferred from the nutritional phenotype of each transformant, we prepared genomic DNA from each heterocaryon and from its component homocaryons. Southern blots of *Cla*I digests of each were prepared and hybridized to probes representative of each of the transforming plasmids to determine the presence or absence of integrated plasmid DNA. The identity of the integrated plasmids for each isolate coincided completely with predictions based on the nutritional requirements. Most isolates displayed hybridization patterns indicating more than one site of integration. However, for each particular isolate there appeared to be at least one common *Cla*I fragment hybridizing to both probes. This phenomenon was further studied by digesting intact chromosomal DNA with *Not*I, an enzyme for which there is no recognition site in either of the transforming plasmids. The digested samples were run on pulsed-field CHEF gels (CHU *et al.* 1986) and Southern blots of the gels were probed with inserts from each of the plasmids. Common fragments were revealed by the two different probes (see Figure 3). These results indicate that the two transforming plasmids had at least one common site of integration. This confirms that it was a single nucleus that was trans-

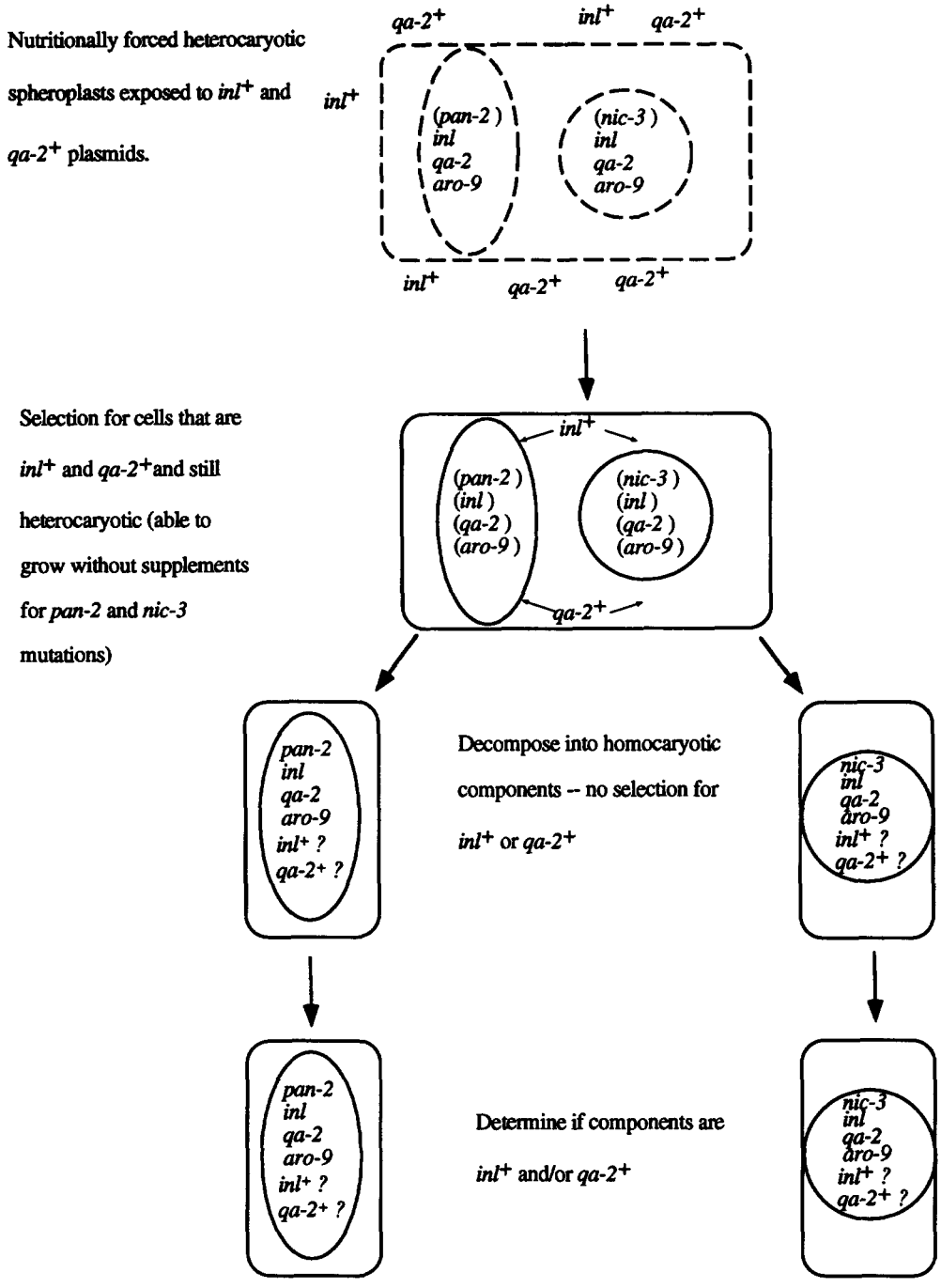

FIGURE 2.—Diagram depicting one competent, heterocaryotic spheroplast and how the nature of the transformation events that allowed it to grow were determined. The two nuclear types are represented by an oval (*pan-2*) and a circle (*nic-3*). Only the genetic markers involved with nutritional selection in this experiment are shown (for a complete description see MATERIALS AND METHODS). Alleles within parentheses were kept under selection at the indicated stages by omission of the required nutrients. Expression of the transforming plasmids was required only in the initial selection. After that, selection was relaxed and the homocaryotic components were isolated. These were then checked for the presence or absence of each plasmid by determining growth requirements.

formed (not two with identical genotypes at the moment before transformation). It also suggests that there could be privileged or "competent" chromosomal sites for ectopic integration; thus the term "random" could be misleading when used to describe ectopic integration.

Plasmids appear to be integrated as separate entities: The genetic linkage of ectopically integrated, cotransformed DNA sequences has been observed before and it has been suggested that the heterologous DNA sequences are ligated and exist as a common structure before integration (PERUCHO *et al.* 1980; ROBBINS *et al.* 1981; REID *et al.* 1991). It seemed possible that in our experiment the two plasmids were covalently joined ei-

TABLE 2

Results of resolution of heterocaryotic, cotransformed isolates

Class	Genotypes	No. observed
	nic-3 nucleus: untransformed	
I	*pan-2* nucleus: transformed by *qa-2*$^+$, *inl*$^+$	25
	nic-3 nucleus: transformed by *qa-2*$^+$, *inl*$^+$	
I	*pan-2* nucleus: untransformed	24
II, III	Others (see Table 1)	0
	nic-3 nucleus: transformed by *qa-2*$^+$, *inl*$^+$	
III	*pan-2* nucleus: transformed by *qa-2*$^+$	1
	nic-3 nucleus: transformed by *qa-2*$^+$, *inl*$^+$	
IV	*pan-2* nucleus: transformed by *qa-2*$^+$, *inl*$^+$	2

ther in the cytoplasm or the nucleus and thus affected the outcome of our experiment. In an effort to detect the presence of any coligated plasmids we treated spheroplasts with the mixture of transforming plasmids as usual, and then harvested the cells at three different stages: immediately after transformation, 16 hr after transformation (at which time the cell walls had regenerated) and after allowing growth for an additional 24 hr after regeneration. In each case, the cells were lysed and DNA was isolated. The DNA samples were examined without digestion for the presence of free plasmids, both by transformation of bacterial cells and by Southern analysis. While the individual plasmids could be found by both techniques, no coligated ones whatsoever were found. It appears that the plasmids do not exist as free covalently joined molecules, though the possibility cannot be formally excluded that they are coligated but have only a very transient free existence between coligation and integration.

Some treatments that disrupt chromosome structure have no effect on efficiency of transformation: Our results indicated that some feature(s) at the level of the nucleus determines competence for transformation and that one of these features may be the availability of an appropriate chromosomal site for integration. A preexisting break in the chromosome could be considered to be such a site. If this hypothesis is correct, it might be possible to radically increase the proportion of available sites, and therefore competence, by treatments which induce breaks in chromosomes.

We used two methods to create breaks in the chromosomes. One method involved the use of ultraviolet light, which has been shown to cause chromosomal rearrangements in Neurospora (PERKINS 1974). Spheroplasts were prepared and allowed to take up plasmids as usual, but before they were allowed to regenerate cell walls, they were subjected to graded doses of ultraviolet light up to 75% killing. The suspensions were plated to selective medium and observed for differences in transformation efficiency in comparison to that of unirradiated cells. No significant difference was seen.

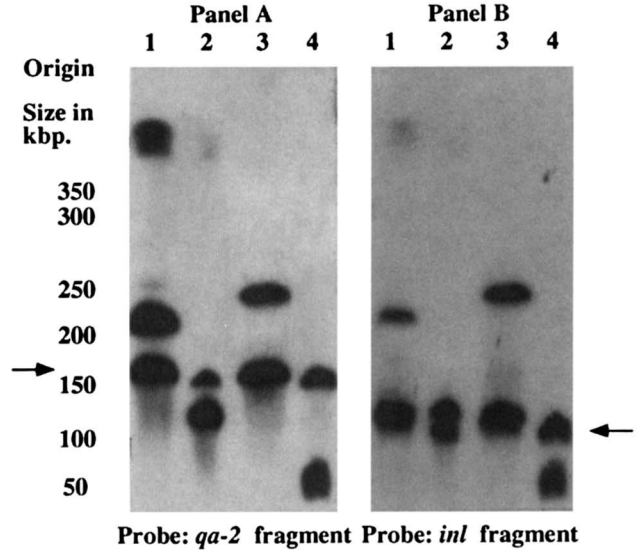

FIGURE 3.—Autoradiograms of a single Southern blot from a CHEF gel. Intact chromosomal DNA was obtained from four different, cotransformed, homocaryotic components of heterocaryons that were isolated according to the experimental procedure previously described (see Figure 2). The DNAs were digested with the restriction enzyme *Not* I and the fragments were separated by electrophoresis as described in MATERIALS AND METHODS. (A and B) The same blot; the blot was initially hybridized to a *qa-2* probe and exposed to film to produce A. It was then stripped, hybridized with a probe for *inl*, and exposed to film to produce B. A band representative of the endogenous copy of the gene being used as a probe (indicated by arrows) and at least one band representative of the ectopically integrated transforming DNA was present in each isolate.

Another method involved the use of an unstable partial diploid containing a duplicated segment of Linkage Group I fused to the nucleolus organizer on Linkage Group V. This duplication, derived from the translocation T(I → V)AR190, spontaneously and rapidly loses the entire ectopic segment of duplicated DNA in most of the nuclei and does so in the rest of them as well unless one or more alleles in the ectopic segment are held under selection (BARRY and PERKINS 1969; PERKINS and BARRY 1977; BUTLER and METZENBERG 1990). The breakage occurs at random or nearly random sites within the nucleolus organizer, and the breakpoint is soon "healed" by *de novo* addition of a telomere (BUTLER 1992). We prepared a strain containing the AR190-derived duplication, with suitable forcing markers on the ectopic and endogenous segments of LG I to maintain the partial diploidy in some nuclei. The strain also carried *qa-2;aro-9* and therefore required aromatic amino acids. Conidia from this strain were germinated in medium which no longer selected for maintenance of the duplication. Spheroplasts were made from these germinated conidia, some of which will contain a fresh break at the end of the chromosome terminating in the nucleolus organizer (BUTLER 1992). These were then transformed to independence for aromatic amino acids

with the pRAL-1 plasmid, carrying *qa-2*$^+$. It seemed possible that the breaks would act as sites of plasmid integration and that this might both increase the frequency of transformation and cause the integrated plasmids to be contiguous to rDNA of the nucleolus. No significant increase was observed in the proportion of spheroplasts that was competent. Twenty of the transformants were further analyzed by restriction digestion followed by CHEF gel electrophoresis (BUTLER and METZENBERG 1989) to determine whether the transforming plasmid DNA had integrated adjacent to rDNA. None of the isolates studied displayed such a pattern of integration.

Our attempts to create a so-called competent chromosomal site for integration of transforming DNA were unsuccessful. This does not disprove the hypothesis that a preexisting break in the chromosome plays a role in competence. It has been previously reported that transforming DNA does integrate at sites of translocation (ASCH *et al.* 1992; PERKINS *et al.* 1993) and it has been suggested that ectopic integration does occur at preexisting breaks (ROTH and WILSON 1988). It is likely that there are other determining factors involved with integration, and that the detailed chemistry at the breakpoint is important (SCHIESTL *et al.* 1993).

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