Curr Genet (1988) 13:65–70

Current Genetics

Transformation of *Neurospora crassa* with the *trp-1* gene and the effect of host strain upon the fate of the transforming DNA

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Summary. *Neurospora trp-1*$^+$ transformants, obtained by transforming a *trp-1 inl* strain with plasmid DNA containing the wild type *trp1*$^+$ gene, were characterized by genetic and Southern blot analyses. The transforming *trp-1* gene integrated at or near the resident site in all of the *trp-1*$^+$ transformants obtained with circular DNA or DNA cut within the *trp-1* coding region. The frequency of homologous integration decreased substantially when the donor DNA was cleaved outside the *trp-1* coding region. The transformants were very stable mitotically and, in general, also showed meiotic stability. Analysis of *trp-1*$^+$ transformants obtained with another recipient strain, *trp-1*$^+$ *ga-2 aro-9 inl*, showed that homologous integration of donor DNA occurred in only 20% of the transformants, whether circular or linear DNA was used. Thus, the host strain employed for transformation appears to be a major factor in determining the fate of transforming DNA. Southern blot analysis of transformants showed that integration of the transforming DNA at the homologous site occurred by double crossover or gene conversion events rather than by insertion of the entire plasmid DNA. Multiple and apparently non functional integration events were observed in some transformants.

Key words: *Neurospora* transformation – *trp-1* gene

Introduction

The molecular cloning of genes of many different organisms has led to important new insights concerning gene structure and expression. In some instances, isolated genes can be subjected to in vitro manipulations and returned to the original host by transformation, where the effect of precisely defined genetic alterations upon gene expression and regulation can be studied. An important feature in such approaches is the status of the transforming DNA, whether it remains as a freely replicating entity or is integrated into the host genome. Moreover, when transforming DNA is integrated rather than having it inserted at random sites in the genome, it is valuable to be able to target it to its normal genomic location and to be able to carry out precise gene disruptions.

In the fungus *Neurospora crassa*, transformation with cloned genes is readily achieved (Case et al. 1979; Dhawale et al. 1984). However, several studies with *Neurospora* have indicated that the transforming DNA very frequently integrates at secondary sites rather than at the homologous region of the genome (Case et al. 1979; Case 1986; Dhawale and Marzluf 1985; Paietta and Marzluf 1985). In a recent analysis of *ga-2*$^+$ transformants, only 5% of the transformants were of the linked type, where the transforming *ga-2*$^+$ gene integrated at its homologous site (Dhawale and Marzluf 1985). Linearization of the donor DNA led to a moderate increase in the transformation frequency but did not affect the proportion of linked transformants. In the case of the *am* gene, the fate of the transforming DNA appeared to depend on the vector DNA. Transformants obtained with the *am*$^+$ gene carried on a λ phage vector were stable meiotically, and up to 40% of them were of the linked type (Kinsey and Rambosek 1984). However, when subcloned into a plasmid vector and employed for transformation, the *am*$^+$ gene came through crosses extremely poorly, suggesting that the transforming plasmid DNA might exist as an autonomously replicating entity (Grant et al. 1984).

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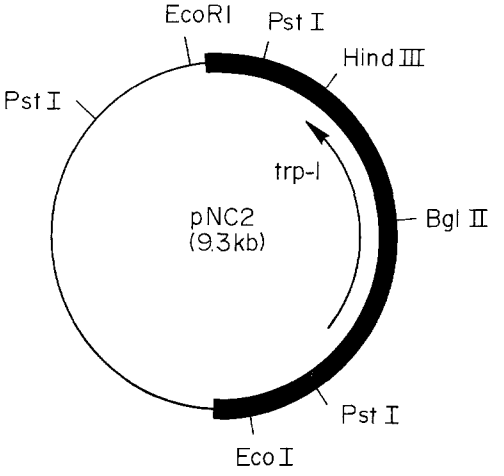

Fig. 1. Schematic diagram of pNC2. The *thin line* represents the sequence derived from pBR322. The *thick line* indicates *Neurospora* DNA containing the *trp-1* gene (5 kb). The *arrow* shows the coding region and direction of transcription. pNC2 has no *SmaI* site. Only relevant restriction sites are shown

In *Aspergillus nidulans,* the degree of homologous insertion of transforming DNA varies, depending upon the selective marker and the host strain, although in some cases a high degree of targeting was achieved (Yelton et al. 1984). In the yeast, *Saccharomyces cerevisiae,* essentially all integration of donor DNA appear to occur by homologous recombination, although the transformation frequency varies with host strain (Orr-Weaver et al. 1981). Transforming DNA cut within the yeast sequences greatly increases the efficiency of integration, up to several thousand fold, depending on donor DNA.

It is not clear yet whether the behavior observed with the *ga-2* or the *am* genes will prove to be a general feature of transformation in *Neurospora,* because extensive studies on transformation with other *Neurospora* genes have not been reported. In this paper we report a detailed study of *Neurospora trp-1*$^+$ transformants and the fate of the transforming DNA. We present results which show that targeting of the transforming DNA to its normal resident locus in the genome can be significantly influenced by the host strain, and show that in one case, perfect targeting of *trp-1* transforming DNA was achieved.

Materials and methods

Neurospora strains and transformation. Transformation of *Neurospora* was performed by the lithium acetate protocol (Dhawale et al. 1984). Recipient strains were *trp-1 inl* (alleles 20 and 37401, respectively) and *trp-1 ga-2 aro-9 inl* (alleles 25, M246, M6–11, and 4701, respectively). When linear plasmid DNA was employed for transformation, the entire restriction digestion mixture was used without any purification of restriction fragments. Genetic analysis was performed with homokaryotic transformants isolated by crossing primary transformants with the *trp-1* (25) strain. The *trp-1 (25) ad-2* strain was constructed by crossing the *trp-1 (25)* strain with the *ad-2* strain and *ga-2 aro-9 trp-1 inl* was constructed by crossing the *ga-2 aro-9 inl* strain with the *trp-1* strain.

Genetic analysis of transformants. Homokaryotic *trp-1*$^+$ transformants were crossed with the *trp-1 (25) ad-2* strain and 40 to 50 randomly-chosen *trp-1*$^+$ progeny from each cross were tested for an adenine requirement for growth. The *ad-2* locus is closely linked to the *trp-1* locus (1–7% recombination), and therefore nearly all of the *trp-1*$^+$ progeny are expected to be *ad-2*$^+$ if the transforming *trp-1* gene had integrated at or near the *trp-1* site, while 50% of the *trp-1*$^+$ progeny will be *ad-2*$^+$ if the gene had integrated at an unlinked site.

Preparation of DNA and Southern blot analysis. Plasmid DNA was prepared according to Birnboim and Doly (1979). *Neurospora* DNA was isolated by a modification of Metzenberg and Baisch's procedure (1981). For Southern blot analysis 30 μg of genomic DNA was digested with 90 units of *SmaI* for 7 to 8 h. Southern transfers, hybridizations, and preparations of radioactively labeled probes by nick translation were performed according to Maniatis et al. (1982).

Materials. Plasmid pNC2 was a gift from Dr. M. Schechtman. Restriction enzymes were purchased from International Biotechnologies, Inc. or from Bethesda Research laboratories. ^{35}S-dCTP was from New England Nuclear Corp. Nitrocellulose paper was obtained from Schleicher and Schuell.

Results

Genetics analysis of trp-1$^+$ *transformants*

Transformation of trp-1 (20) inl strain. The *trp-1* gene encodes a polypeptide which has three different enzyme activities involved in the biosynthesis of tryptophan: anthranilate synthase (AS), indoleglycerolphosphate synthase (IGPS), and phosphoribosylanthranilate isomerase (PRAI) (Schechtman and Yanofsky 1983). The *trp-1 inl* recipient strain has a near normal level of AS function and partial IGPS activity, but is completely missing PRAI activity (DeMoss et al. 1967). Therefore, the host strain requires tryptophan for growth. The nature of the mutation has not been reported. However, the *trp-1 (20)* mutant did not produce any revertants upon UV treatment (DeMoss et al. 1967) suggesting that the mutation is very stable.

The *trp-1* mutant strain was transformed with plasmid pNC2 DNA, which carries the entire *trp-1* gene (Fig. 1), and both circular and linear DNAs were used. pNC2 DNA was treated with three restriction enzymes, *HindIII, PstI,* and *BglII. HindIII* cuts the plasmid DNA only once, immediately 3′ to the *trp-1* coding region, a single *BglII* site occurs in the middle of the gene, whereas *PstI* cuts the plasmid at three sites, before and

Table 1. Genetic analysis of *trp-1*$^+$ transformants. Transformants were analyzed as described in Materials and methods

DNA	No. analyzed	No. of each type		
		Linked	Unlinked	Unclassified
Recipient strain: *trp-1 inl*				
pNC2 circular	10	10	0	0
pNC2 linear (*BglII*)	10	10	0	0
pNC2 linear (*HindIII*)	9	5	3	1
pNC2 linear (*PstI*)	10	6	3	1
Recipient strain: *trp-1 ga-2 aro-9 inl*				
pNC2 circular	8	1	5	2
pNC2 linear (*HindIII*)	7	2	2	3
pAMC2 circular	5	1	4	0
pAMC2 linear (*HindIII*)	9	2	6	1

after the *trp-1* gene and once in the pBR322 sequence of pNC2.

Trp-1$^+$ transformants were obtained at a frequency of about 4/μg DNA with circular or *BglII*-cut plasmid DNA, while a several fold higher transformation rate was obtained with *HindIII* or *PstI*-digested DNA. In a control experiment, in which DNA was omitted, no *trp-1*$^+$ colonies were obtained. Primary transformants, which are heterokaryons, varied widely in their growth pattern. Some formed extremely small colonies on the initial selective medium, while others grew vigorously and formed large colonies. However, more than 95% of the transformants continued to grow upon transfer onto fresh medium. Thus, abortive transformants, which were observed at a high frequency in *ga-2*$^+$ or *am* transformants, were only a minor class among *trp-1*$^+$ transformants, implying that the transformed *trp-1*$^+$ gene was stably integrated.

Genetic analysis of transformants. To examine whether the *trp-1* gene had integrated into its resident site or not, homokaryotic transformants were crossed with the *trp-1 ad-2* double mutant strain and progeny from 39 such crosses were analyzed as described in Materials and methods. As shown in Table 1, all of the 10 transformants examined which were obtained with circular pNC2 DNA were of the linked type, in which the *trp-1*$^+$ gene cosegregated with the *ad-2* marker. No unlinked transformants were observed, indicating that the transforming *trp-1*$^+$ gene integrated at or near the resident locus in all of these transformants. Similarly, only linked transformants were obtained with BglII-cut DNA, as expected, since *BglII* cuts within the *trp-1* coding region.

The proportion of linked type transformants was somewhat lower when linear pNC2 DNA obtained by digestion with *HindIII* or *PstI* was used; 11 of 19 transformants examined were of the linked type. In 6 transformants the *trp-1*$^+$ gene segregated independently from the *ad-2* marker, indicating that the transforming DNA had integrated at genomic sites unlinked to the resident *trp-1* gene. The remaining 2 transformants could not be clearly classified as linked or unlinked. Thus, linearization of transforming DNA resulted in an overall increase in transformation rate but a decrease in the frequency of homologous integration.

Mitotic stability of transformants. 11 homokaryotic transformants were randomly chosen and, after 10 serial transfers to a non-selective medium to enrich for any *trp-1* mutant cells which might arise as a result of mitotic instability, conidia were plated on the same medium. 96 colonies from each transformant were then tested for their viability on selective medium. Eleven of the transformants, which included 6 of the linked type and 5 unlinked ones showed complete mitotic stability in this test (results not shown).

Meiotic stability of transformants

In crosses involving transformants, considerable differences in the recovery of *trp-1*$^+$ progeny were observed, suggesting variation in the meiotic stability of the transformed gene. Therefore, it seemed important to determine whether or not the transformed *trp-1*$^+$ marker was stable during meiosis. Ascospores from crosses between transformants and the *trp-1*$^+$ *ad-2* strain were plated on a non-selective medium, and individual colonies were tested for growth on minimal medium. If the *trp-1*$^+$ gene passed through a cross in a stable manner, 50% of the progeny should be *trp-1*$^+$.

The results of this meiotic stability test are presented in Table 2. In 7 of 10 crosses of linked transformants *trp-1*$^+$ progeny were recovered at the expected frequency of 50% (or at a frequency comparable to that of control crosses). However, *trp-1*$^+$ progeny occurred at a much lower frequency in the remaining 3 crosses. In the case of unlinked transformants, the portion of *trp-1*$^+$ progeny was similar to that observed with control crosses in 6 of 8 such crosses; in the other two crosses, only 3% of the progeny were *trp-1*$^+$. In summary, the transformed *trp-1*$^+$ gene segregated as expected for a stable marker in crosses involving most (13 of 18) of the transformants analyzed. Thus, most *trp-1*$^+$ transformants were stable meiotically, although 5 instances of obvious instability were observed.

Transformation of ga-2 aro-9 trp-1 inl strain. The experiments described above revealed that the characteristics of *trp-1*$^+$ transformants differ from the previously-

Table 2. Meiotic stability of *trp-1*$^+$ transformants. Each control and each transformant were crossed with the *trp-1 ad-2* mutant strain and progeny were tested to determine the rate of segregation of the *trp-1*$^+$ gene through the cross

Transformant	No. of progeny tested	No. of *trp-1*$^+$ progeny	%*trp-1* progeny
Control crosses			
Wild type A	60	24	40
wild type a	60	16	27
inl a	60	28	47
Linked transformants			
1-60-1	48	17	35
1-72-1	60	17	28
1-100-3	48	25	52
2-7-4	48	13	27
2-34-1	48	22	46
2-45-4	48	23	48
2-64-2	96	6	6
2-82-2	60	1	2
2-89-3	48	19	40
2-91-3	60	6	10
Unlinked transformants			
2-4-3	96	3	3
1-14-1	48	14	29
2-19-2	48	9	19
2-25-4	48	26	54
2-44-2	48	9	19
2-55-1	48	10	21
2-57-1	48	19	40
2-68-4	96	3	3

studied *ga-2*$^+$ and *am*$^+$ transformants. The most significant difference is the decidedly higher frequency of the linked type of transformants obtained with *trp-1* compared with *ga-2* or *am*. Moreover, most *trp-1*$^+$ transformants showed meiotic stability, in contrast to *ga-2*$^+$ or *am*$^+$ transformants. These differences might be due to different properties of the recipient strain or, alternatively, due to different characteristics of the transforming DNA.

In order to examine the possiblity that the recipient strains could dramatically affect the results of transformation, particularly the frequency of targeting of the transforming DNA, we studied transformation with the *trp-1*$^+$ gene of a second host strain. This second host strain, *ga-2 aro-9 trp-1 inl*, was derived from a cross of the *trp-1* mutant with the triple mutant strain, *ga-2 aro-9 inl*, this latter strain has been widely used for transformation with the *ga-2* gene, which targeted very poorly to its resident locus (Case 1986; Dhawale and Marzluf 1985). This second strain was transformed with the same plasmid pNC2 DNA, and homokaryotic *trp-1*$^+$ transformants were analyzed genetically to determine the integration site of the *trp-1* gene. The results, summarized in Table 1. show that transformation of this new host strain with circular pNC2 DNA yielded only 12.5% (1 of 8 tested) transformants of the linked type; 5 transformants were unlinked, while 2 could not be readily assigned to either class. The proportion of linked transformants was also very low among transformants obtained with *HindIII*-cut pNC2 DNA.

The results were significantly different from those obtained by transforming the *trp-1 inl* host. The linked type of *trp-1*$^+$ transformants were much less frequent and, transformants that showed abnormal meiotic behavior and thus could not be classified as either a linked or an unlinked type were observed at a higher frequency. This outcome indicates that the host strain for transformation is an important factor in determining the fate of transforming DNA.

In a separate experiment, *trp-1*$^+$ transformants were obtained with the same *ga-2 aro-9 trp-1 inl* recipient strain with a plasmid DNA (pAMC2) containing both the *trp-1* and the *ga-2* gene and were analyzed as described above. It was anticipated that the frequency of linked *trp-1*$^+$ transformants might be lower due to the *ga-2* segment promoting integrating at other sites. However, as shown in Table 1. the addition of the *ga-2* sequence made no difference in the integration pattern of the *trp-1*$^+$ gene, implying that the DNA segment itself did not strongly affect the targeting of the transforming DNA.

Southern blot analysis of transformants

The genetic analysis indicated that the *trp-1* gene was integrated into its homologous site in 80% of the transformants obtained from the *trp-1 inl* recipient strain. To investigate the integration sites at the molecular level, 15 *trp-1*$^+$ transformants (from the *trp-1 inl* host) were examined by Southern blot analysis. If the linked type transformants arose by precise replacement of the resident sequence by the transforming DNA, only the resident band should be detected in a Southern blot. On the other hand, the resident band will disappear and another band will show up at an altered position if integration occurred by insertion of the whole donor DNA.

As shown in Fig. 2, the resident *trp-1* band was detected in an unaltered form in all of the linked transformants examined, and in no case was an altered resident band observed. The resident band in these linked transformants did not hybridize with a pBR322 probe, showing that no vector sequences had integrated. Thus, these transformants seem to have occurred by precise replacement rather than by insertion via a single crossover, the major route of integration in yeast or *Aspergillus*. Since double crossover events are less frequent

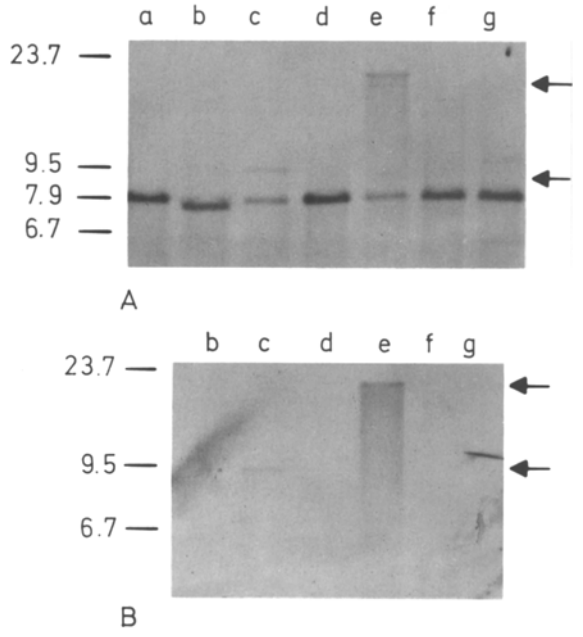

Fig. 2. Southern blots of linked transformants. *SmaI*-digested genomic DNA of the wild type strain, the recipient strain, and 5 linked transformants was electrophoresed, transferred to nitrocellulose papers, and probed with labeled *HindIII/PstI* fragment of pNC2 containing the *trp-1* gene (*panel* **A**) or with labeled pBR322 (*panel* **B**). *Lanes: a,* wild type; *b,* recipient strain; *c,* 1-60-1; *d,* 1-100-3; *e,* 2-34-1; *f,* 2-45-4; *g,* 2-89-3. *Arrows* indicate the position of an additional band in transformants 1-60-1 and 2-34-1. Sizes are given in kb

than single crossovers, it is likely that integration of *trp-1* gene actually occurred via gene conversion.

In two transformants (1-60-1) and (2-34-1) one additional band also hybridized with the *trp-1* probe, indicating that a second integration event had taken place. Both of these additonal bands also responded to the pBR322 probe. The genetic analysis, however, suggested that neither of these additional bands were functional. The weaker intensity (one fourth that of the resident band) of the additional band in transformant 1-60-1 suggested that only part of the *trp-1* sequence was inserted in the second integration event. The size of the band (9.3 kb) further supported the possibility of deletion of part of the transforming DNA since DNA fragments larger than pNC2 (9.3 kb) would be expected in Southern blots of *SmaI*-digested genomic DNA.

Southern blots of unlinked transformants are presented in Fig. 3. As expected, at least one additional band was observed in each of these transformants. All of the extra bands hybridized with both the *trp-1* and the pBR322 probes, showing that both *trp-1* DNA and vector sequences were integrated into the genome. The size of the additional bands were variable but always greater than that of pNC2. Thus, no preferred secondary integration site could be discerned by the Southern blot analysis. Integrations at multiple unlinked sites were not common in the case of *trp-1* transformants. Only in one (2-68-4) of 8 unlinked transformants were two nonresident bands detected. Transformant 2-4-3, which was very unstable meiotically, and could not be easily assigned as either a linked or an unlinked transformant by genetic analysis, appears to be an unlinked transformant by the Southern blot analysis. Because the intensity of the additional band was about twice that of the resident band, two copies of the *trp-1* sequence may have integrated at a non-homologous site in this transformant. This could be the reason for the meiotic instability of this transformant.

Two linked transformants (2-64-2 and 2-82-2), according to genetic analysis, which were meiotically unstable, are also included in Fig. 3. Each of them possessed one additional band of similar intensity to the resident band; in each case the extra band also hybridized with the pBR322 probe. It is conceivable that the donor DNA integrated near but not at the resident *trp-1* locus, resulting in duplication of the *trp-1* sequence at a nearby site. Meiotic instability could then be explain-

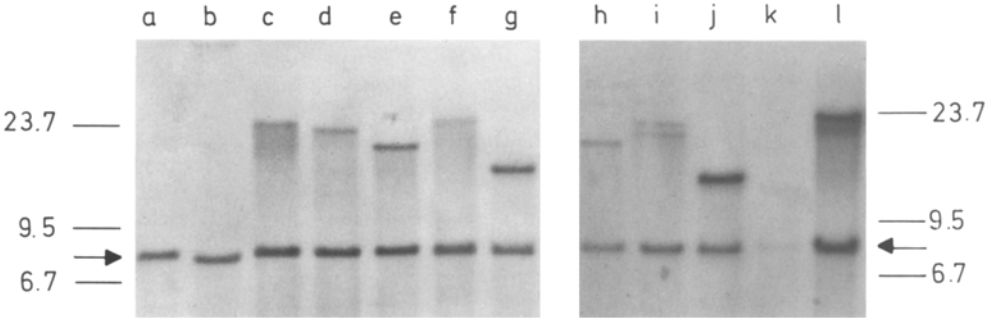

Fig. 3. Southern blots of transformants. *SmaI*-digested genomic DNA of the wild type strain, the recipient strain, 8 unlinked transformants, and 2 linked transformants was electrophoresed, transferred to nitrocellulose papers, and probed with labeld *HindIII/EcoRI* fragment of pNC2 containing the *trp-1* gene. *Lanes: a,* wild type; *b,* recipient strain; *c,* 2-14-1; *d,* 2-19-2; *e,* 2-25-4; *f,* 2-44-2; *g,* 2-55-1; *h,* 2-57-1; *i,* 2-68-4; *j,* 2-4-3; *k,* 2-64-2; *l,* 2-82-2. The *arrow* shows the position of the resident band. Sizes are given in kb

ed by frequent loss of *trp-1* function, possibly by recombination between duplicated sequences during meiosis, or by rearrangement and methylation of duplicated sequences as shown by Selker and Stevens (1987).

Discussion

Previous studies on the transformation of *Neurospora crassa* showed that integration of transforming DNA into its homologous site occurred infrequently, unlike the situation in other closely related fungi. The frequency of homologous integration in *Neurospora* appears to lie between that of yeast and of mammalian cells in which it occurs rarely (Thomas et al. 1986).

The analysis of transformants obtained with circular DNA with the first host strain were surprising; the *trp-1*$^+$ gene showed linkage to the *ad-2* locus (and thus to its resident site) in all of transformants examined. This result was similar to high degree of targeting obtained with yeast and *Aspergillus,* rather than the frequent unlinked insertions found with other *Neurospora* genes.

To investigate the possible influence of the host strain upon the integration pattern of transforming DNA, we compared transformation of the identical *trp-1*$^+$ gene into two different *trp-1* mutant host strains. With circular pNC2 DNA, 100% of the *trp-1*$^+$ transformants had targeted to the resident locus with one host strain, whereas only 12.5% targeting was observed with the second host. Thus, it is clear that the host strain utilized for transformation can be significant and strongly influence the degree of targeting achieved.

The marked difference in the proportion of linked transformants observed with these two different host strains almost certainly reflects a difference in their genetic backgrounds. In *Neurospora* the frequency of recombination varies widely among various laboratory strains (Baker et al. 1976). Many genes are known to affect recombination frequency; some have a general effect, reducing or abolishing recombination, while others have a regional effect, promoting or repressing crossing over at a particular chromosomal site (Catcheside 1974, 1977). Some of these genes encode nucleases or products related to DNA repair, and thus may influence mitotic recombination as well.

The fact that not a single case was observed of an altered residend band in linked transformants was somewhat unexpected. Both in *Aspergillus* and yeast, the majority (70 to 80%) of the homologous integration occurs by a single crossover event, resulting in the insertion of the entire plasmid DNA and thus an alteration in resident band. However, our results showed that such an integration event had not taken place in any of the transformants analyzed, indicating that *trp-1* gene integrated by other means.

Our results showed that homologous integration of transforming DNA can occur at a high frequency in *Neurospora,* and appear to depend upon the host strain employed for transformation. Recently Akins and Lambowitz (1985) and Vollmer and Yanofsky (1986) have developed efficient sib-selection methods for cloning *Neurospora* genes by complementation, and virtually any gene can be cloned if a corresponding mutant is available. However, efforts to develop autonomously replicating shuttle vectors for *Neurospora* have not been successful so far. Thus, it is necessary to find a way to "target" an in vitro manipulated gene to specific chromosomal sites to study the expression of *Neurospora* genes in detail. Our work indicates that selection of a suitable host strain is very important to accomplish that purpose.

Acknowledgments. We thank Dr. Michael Schechtman for plasmid pNC2 and Anna Tuton for plasmid pAMC2. This research was supported by grant GM-23367 from the National Institute of Health and by grant 85-CRCR-1-1556 from the US Department of Agriculture.

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Communicated by C. P. Hollenberg

Received May 21, 1987