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General Method for Cloning *Neurospora crassa* Nuclear Genes by Complementation of Mutants

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We have developed a sib selection procedure for cloning *Neurospora crassa* nuclear genes by complementation of mutants. This procedure takes advantage of a modified *N. crassa* transformation procedure that gives as many as 10,000 to 50,000 stable transformants per μg of DNA with recombinant plasmids containing the *N. crassa qa-2*$^+$ gene. Here, we describe the use of the sib selection procedure to clone genes corresponding to auxotrophic mutants, *nic-1* and *inl*. The identities of the putative clones were confirmed by mapping their chromosomal locations in standard genetic crosses and using restriction site polymorphisms as genetic markers. Because we can obtain very high *N. crassa* transformation frequencies, cloning can be accomplished with as few as five subdivisions of an *N. crassa* genomic library. The sib selection procedure should, for the first time, permit the cloning of any gene corresponding to an *N. crassa* mutant for which an appropriate selection can be devised. Analogous procedures may be applicable to other filamentous fungi before the development of operational shuttle vectors.

The filamentous fungus *Neurospora crassa* has been a useful model organism for studies of genetics, metabolic regulation, and development. Procedures for genetic analysis of *N. crassa* are well developed, and large numbers of nuclear mutants have been isolated and mapped (17). *N. crassa* nuclear genes have been cloned by several methods, including complementation of specific *Escherichia coli* mutants (2, 9, 19, 25), screening of genomic libraries by hybridization with DNA probes (14, 27), and screening of cDNA libraries by using antibodies to specific proteins (1, 26). Case and co-workers (4, 5, 20) have developed an efficient transformation procedure for *N. crassa*, using recombinant plasmids containing the *N. crassa qa-2*$^+$ gene. Several alternative *N. crassa* transformation procedures have been described recently (3, 8). Thus far, however, procedures for cloning nuclear genes by complementation of *N. crassa* mutants have been lacking.

In yeasts, nuclear genes corresponding to specific mutations have been isolated by transforming mutants with plasmid libraries of wild-type DNA and then recovering integrated or autonomously replicating plasmids by retransformation of *E. coli* (24). Integrative transformation of *N. crassa* is now a well-established procedure. However, at most 30% of the stable integrants obtained with recombinant plasmids containing the *N. crassa qa-2*$^+$ gene retain the integrated transforming gene linked to bacterial plasmid sequences which are essential for recovery in *E. coli* (4). In addition, we have observed some transformants in which "nonfunctional" plasmid sequences have integrated repeatedly at different chromosomal locations (10). Possibly as a result of these complications, cloning by plasmid rescue from *N. crassa* integrative transformants has not yet been reported.

We recently reported that certain recombinant plasmids appear to replicate autonomously in both *N. crassa* and *E. coli* (10, 22, 23). However, these plasmids are present in relatively low copy number and are subject to alterations, including large deletions, in standard *N. crassa* host strains (10, 22, 23). Consequently, it is still unclear whether the presently available plasmids will be useful for routine nuclear gene isolations in *N. crassa*.

On the other hand, an important advantage of the *N. crassa* transformation system is that it has been possible to obtain very high transformation frequencies (10,000 to 50,000 stable transformants per μg of DNA) with recombinant plasmids containing the *N. crassa qa-2* gene. These high transformation frequencies suggested to us that it might be possible to clone *N. crassa* genes rapidly by sib selection, i.e., repeated subdivision of a genomic library, progressively enriching for the transforming gene. Sib selection cloning procedures have been used previously, for example, to isolate sea urchin histone genes (13) and the yeast *his-4* gene (11). Here, we describe the development of a rapid sib selection cloning procedure for *N. crassa* and the use of this procedure to clone genes corresponding to the auxotrophic mutants, *nic-1* and *inl*. This sib selection procedure should for the first time permit the cloning of any *N. crassa* nuclear gene corresponding to a mutant for which an appropriate selection can be devised. Analogous procedures may be applicable to other filamentous fungi before the development of operational shuttle vectors.

MATERIALS AND METHODS

Strains of *N. crassa*. The *N. crassa* strain used for transformation experiments was RAA-1*A*, *nic-1* (3416); *inl* (89601); *qa-2* (M246); *aro-9* (M6-11); *al-2* (15300). This strain was constructed by standard crosses. Strains used for mapping restriction site polymorphisms were: Mauriceville-1c *A* (FGSC no. 2225), RLM1-33*a cot-1 al-2 arg-12 nuc-2 inl* (FGSC no. 4411; derived from Oak Ridge), and RLM47-34*a nuc-1 ad-9 al-2 nic-1* (derived from Oak Ridge). RLM1-33*a*, Mauriceville-1c, and selected progeny from a cross between these strains (FGSC no. 4411 through 4430) were obtained from the Fungal Genetics Stock Center (Humboldt State University, Arcata, Calif.). RLM47-34*a* and selected progeny from a cross between this strain and Mauriceville-1c were obtained from Robert L. Metzenberg, University of Wisconsin, Madison, Wis.

Recombinant plasmids. The plasmid used for library construction, pRAL1 (see Fig. 1), consists of the *N. crassa*

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qa-2$^+$ gene cloned in a shortened derivative of pBR329 (7). To construct this plasmid, pBR329 was linearized with *Pst*I, digested with nuclease BAL 31 to remove approximately 1 kilobase (kb), and religated. The *N. crassa qa-2*$^+$ gene (1,225 base-pair *Bam*HI-*Bgl*II fragment) was isolated from pALS2 (22, 23), treated with S1 nuclease to produce blunt ends, and ligated into the single *Ava*II site, which had also been treated with S1 nuclease to produce blunt ends. The plasmid retains tetracycline resistance (*tet*^{R}) and chloramphenicol resistance (*cap*^{R}) genes which can be selected in *E. coli*.

Isolation of bacterial plasmid DNAs. *E. coli* plasmid DNAs used for transformation of *N. crassa* were isolated by a cleared lysate procedure (15) and purified further as follows. The final cleared lysates were brought to 2 M potassium acetate, placed on ice for 30 min, and centrifuged in a Sorvall GSA rotor (10,000 rpm, 10 min, 4°C). DNAs were recovered in the supernatants, brought to 2 M ammonium acetate, and precipitated by addition of an equal volume of isopropanol and freezing on dry ice. The precipitated DNAs were redissolved in 0.3 ml of 10 mM Tris hydrochloride (pH 8.2)–1 mM EDTA, digested with RNase T1 and A (2,500 U of RNase T1 per ml and 200 μg of RNase A per ml; Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C, and incubated with protease K (200 μg/ml; Sigma) for 30 min at 37°C (protease K was preincubated for 60 min at 37°C). They were then extracted with phenol-chloroform-isoamyl alcohol (25:24:1), brought to 2.5 M ammonium acetate, and precipitated with ethanol. Plasmid DNAs at this stage of purification were used for routine transformation of *N. crassa*. Plasmid DNAs were purified further by centrifugation in cesium chloride-ethidium bromide gradients only when it was necessary to optimize or standardize *N. crassa* transformation frequencies.

E. coli plasmid DNAs for gel analysis were isolated by the above procedures or by the mini-prep procedure (12).

Isolation of high-molecular-weight *N. crassa* DNA and construction of *N. crassa* genomic libraries. DNA for construction of an *N. crassa* genomic library was isolated from wild-type strain 74*A*. Cells were grown to late log phase and collected by filtration on sintered glass funnels. Mycelial pads were ground with sand and incubated in lysis buffer (2% sodium dodecyl sulfate, 50 mM Tris hydrochloride [pH 8.2], 62.5 mM EDTA) for 60 min. The lysates were then centrifuged in a Sorvall GSA rotor (3,000 rpm, 10 min, 4°C) to remove sand and cell debris. The supernatants were brought to 2 M potassium acetate, placed on ice for 30 min, and centrifuged in a Sorvall GSA rotor (10,000 rpm, 10 min, 4°C). The supernatants were recovered and brought to 2 M ammonium acetate. DNA was then precipitated by addition of an equal volume of isopropanol and overnight incubation at −20°C. The DNA pellets were gently redissolved in lysis buffer and purified further by centrifugation in cesium chloride-ethidium bromide gradients. *N. crassa* DNA isolated by this method is larger than 50 kb.

Construction of an *N. crassa* genomic library in pRAL1. pRAL1 was used to construct a recombinant DNA library containing fragments of DNA from *N. crassa* wild-type 74*A*. High-molecular-weight DNA was isolated from this strain as described above, partially digested with *Sau*3A, and size fractionated by sucrose gradient centrifugation essentially as described by Maniatis et al. (15). Fractions containing DNAs of approximately 10 kb were recovered from sucrose gradients, ligated into the single *Bam*HI site of pRAL1, and transformed into *E. coli* strain HB101. To increase the proportion of plasmids having large *N. crassa* DNA inserts, supercoiled plasmids were isolated from the initial *E. coli* transformants, pooled, subjected to a second size fractionation in sucrose gradients, and retransformed into *E. coli* HB101. The library and first-round sib pools were stored both as DNA preparations and as *E. coli* transformants at −70°C.

Transformation of *N. crassa*. The procedure used to transform *N. crassa* is derived from that of Schweizer et al. (20). Although alternative *N. crassa* transformation procedures have been described recently (3, 8), we found that the major difficulty with the original procedure is the variable quality of the enzymes used to digest cell walls. After screening several lots of Glusulase (Sigma), Zymolyase-5000 (Seikagaku Kogyo Co., Ltd., obtained from Miles Laboratories, Elkhardt, Ind.), and Novozym 234 (Novo Laboratories, Wilton, Conn.), we identified a specific lot of Novozym 234 (lot no. PPM 1035) which gives very high frequencies of stable *N. crassa* transformants (10,000 to 50,000 per μg of DNA) with recombinant plasmids containing the *N. crassa qa-2*$^+$ gene. In parallel transformations with pALS1-2 (23), another lot of Novozym 234 (lot no. PPM 1530) was much less efficient (400 stable transformants per μg of DNA), and several lots of Glusulase, the enzyme specified by Schweizer et al. (20), were least efficient (0 to 130 transformants per μg of DNA). In subsequent transformation experiments, the alternate lot of Novozym 234 (lot no. PPM 1530) gave *N. crassa* transformation frequencies that were somewhat higher (1,000 to 4,000 stable transformants per μg of pALS2 DNA) but still 10-fold lower than the optimal lot in the same experiment. For purposes of computing transformation frequencies, we define stable transformants as those which are capable of growing in slants after being picked from the initial selection plates. In different transformations, between 50 and 100% of the colonies picked from plates continued to grow in slants. We did not count or pick microcolonies.

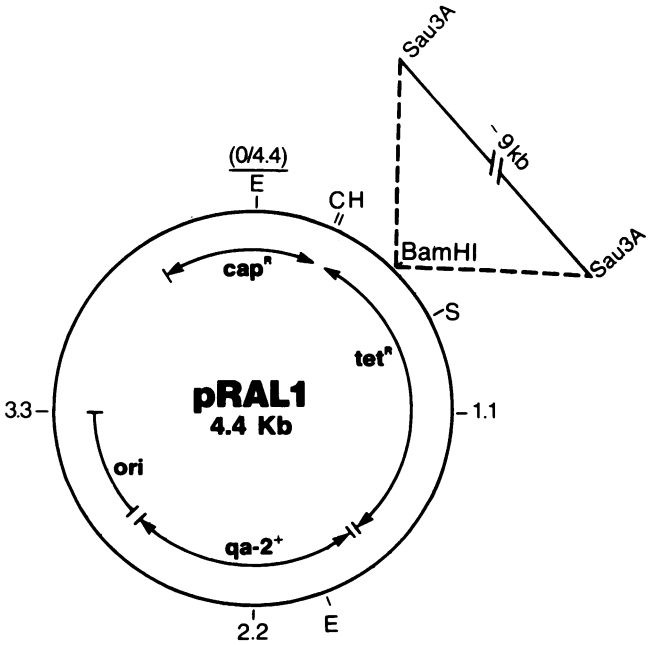

FIG. 1. Partial restriction map of plasmid pRAL1 and the *N. crassa* genomic library. The library was constructed by inserting size-fractionated, *Sau*3A partial digest fragments of DNA from *N. crassa* wild-type 74*A* into the single *Bam*HI site of pRAL1. Restriction sites: E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; S, *Sal*I.

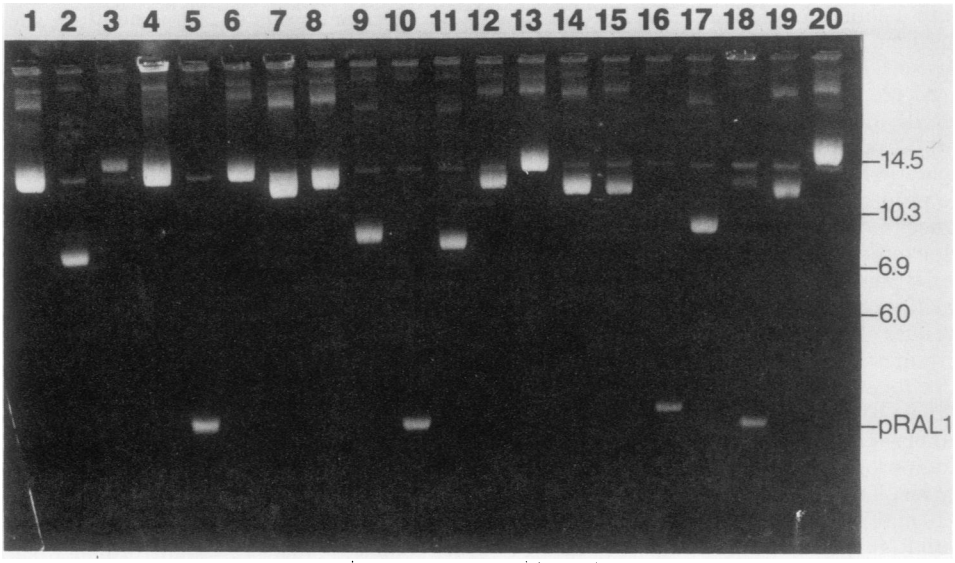

FIG. 2. Gel electrophoretic analysis of 20 random plasmids from the pRAL1 library. DNA was isolated from bacterial colonies by the mini-prep procedure (12) and analyzed by electrophoresis in a 0.8% agarose gel. Molecular weight markers were supercoiled pRAL1 and other previously characterized recombinant plasmids that had been run in parallel lanes of the same gel (not shown).

In our modified transformation procedure, fresh conidia were harvested and inoculated immediately into growth medium (2% sucrose, 0.5× Vogel minimal medium plus appropriate nutritional supplements for auxotrophic mutants). The conidia were allowed to germinate for 4 to 6 h and then collected by centrifugation. To obtain spheroplasts, up to 10^{10} germinated conidia were suspended in 10 ml of 1 M sorbitol and incubated with Novozym 234 (50 mg of unsterilized powder) for 2 h at 30°C. The spheroplasts were then washed and suspended in transformation buffer as described by Schweizer et al. (20). Tests of osmotic stability showed that more than 99% of the conidia were converted to spheroplasts. Between 60 and 100% of the spheroplasts were capable of regenerating to form viable colonies. Transformation efficiencies with pRAL1 generally ranged from 2.5 to 12 transformants per 1,000 viable spheroplasts.

We found that competent spheroplasts obtained after digestion with Novozym 234 and suspension in transformation buffer could be stored frozen in the transformation buffer at −70°C. Such spheroplasts showed no decrease in transformation frequency for at least 1 month and usually much longer. The use of frozen spheroplasts conserves Novozym 234 and facilitates the multiple transformation steps required for the sib selection procedure.

Transformations were carried out at ratios of between 0.2 and 1.2 μg of DNA per 7×10^6 spheroplasts. The highest ratios of DNA to spheroplasts were used with the first-round sib banks to obtain higher numbers of transformants. Maximum transformation frequencies with pRAL1 were obtained over the range of 0.05 to 1 μg of DNA per 7×10^6 spheroplasts. The first rounds of the sib selections were generally carried out with 5 μg of DNA, the second rounds were carried out with 1 μg, and subsequent rounds were carried out with 0.2 μg. The number of spheroplasts plated was adjusted to give a maximum of about 100 transformants per plate.

Most steps in the transformation were essentially as described by Schweizer et al. (20), except that routine *N. crassa* transformations were carried out with *E. coli* plasmid DNAs isolated by the cleared lysate procedure described above. DNAs used in the transformations were purified further by centrifugation in cesium chloride-ethidium bromide gradients only when it was necessary to optimize or standardize *N. crassa* transformation frequencies.

In our experience, the most critical parameters for obtaining very high transformation frequencies were the use of fresh conidia and the specific lot of Novozym 234. We note, however, that even the alternate lot of Novozym 234 (lot no. PPM 1530) still gave sufficiently high transformation frequencies (up to 1,000 to 4,000 stable transformants per μg) to permit sib selection cloning to be carried out readily. In addition, Steven Vollmer, Marc Orbach, and Charles Yanofsky have modified the *N. crassa* transformation procedure to obtain higher transformation frequencies with what we find to be a suboptimal lot of Novozym 234 (lot no. PPM 1530; 10,000 to 40,000 transformants per μg, of which 10 to 20% are stable; Charles Yanofsky, personal communication).

Southern hybridizations. Procedures for gel electrophoretic analysis of DNA and Southern hybridization were as described previously (10, 21). Gels contained 0.8% agarose. Southern blots were hybridized with recombinant plasmids that had been ^{32}P-labeled by nick translation (18).

RESULTS

Characterization of the pRAL1 library. Plasmid pRAL1, which was used for construction of a *N. crassa* genomic library, is shown in Fig. 1. A recombinant DNA library was constructed in pRAL1 by inserting size-fractionated *Sau*3A partial digest fragments of whole-cell DNA from wild-type *N. crassa* 74*A* into the single *Bam*HI site of the plasmid (see above). Gel electrophoretic analysis of 20 plasmids selected at random from the library showed that 80% contain large DNA inserts (Fig. 2). The average size of these inserts, measured by electrophoretic mobility, was approximately 9 kb. To confirm the presence of *N. crassa* DNA sequences, we selected five plasmids at random from the library and hybridized them to Southern blots of *Hin*dIII-*Eco*RI digests of wild-type 74*A* DNA. All the plasmids hybridized to different fragments of *N. crassa* DNA in addition to fragments containing the *qa-2* gene which is present in the vector (data not shown). The pRAL1 library contains more than

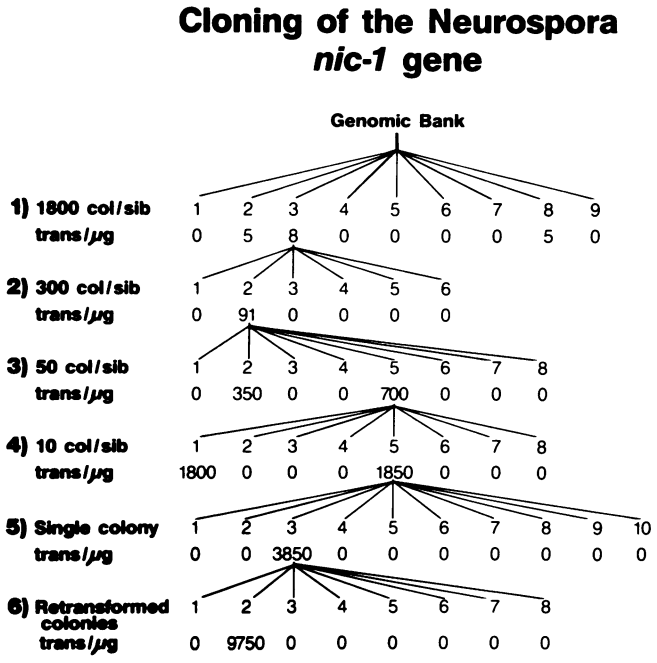

FIG. 3. Cloning of the *N. crassa nic-1* gene by sib selection. In the first transformation step, the pRAL1 library was divided into nine pools (numbered 1 to 9 horizontally) each containing approximately 1,800 plasmids. These plasmid pools were used to transform the RAA-1A *nic-1 inl aro-9 qa-2* host strain. Transformation frequencies (*nic-1*$^+$ *qa-2*$^+$ transformants per μg of DNA; double selection) are shown under the pool numbers. Subsequent transformation rounds are depicted in the same manner. After five subdivisions, the library was reduced to a single colony (pool 3) which contained a mixture of plasmids. This mixture was resolved by retransformation of *E. coli* in round 6.

10,000 independent inserts, which is sufficient to represent the *N. crassa* genome at the 96% confidence level by using the formula of Clarke and Carbon (6). The library gave 4,000 to 6,000 *qa-2*$^+$ transformants per μg of DNA.

Cloning of *nic-1* and *inl* by sib selection. Figure 3 diagrams sib selection cloning of the *N. crassa nic-1* gene. In the first transformation step, the *nic-1 inl qa-2 aro-9* host strain was transformed with nine plasmid pools, each containing about 1,800 plasmids from the pRAL1 library. Three pools (pools 2, 3, and 8) gave significant numbers of *nic-1*$^+$ *qa-2*$^+$ transformants. Bacteria from pool 3, which gave the highest number of transformants, were divided into six pools of 300 colonies for the next transformation step. Subdivision of the library proceeded as diagrammed in Fig. 3. At the fifth round, we obtained a single *E. coli* colony (pool 3) which contained a mixture of at least two plasmids that had to be resolved by an additional transformation step (Fig. 3, round 6). The final *nic-1* clone contained an insert of 6.4 kb and gave 9,750 stable *nic-1*$^+$ *qa-2*$^+$ transformants per μg (double selection) and 14,000 *qa-2*$^+$ transformants per μg (single selection); 10 of 10 *qa-2*$^+$ transformants selected at random were found to be *nic-1*$^+$, as expected.

In the case of *inl*, the initial subpool (pool 7) gave 50 stable *inl*$^+$ *qa-2*$^+$ transformants (double selection) and 6,350 *qa-2*$^+$ transformants (single selection) per μg of DNA. The final clone contained an insert of 14.7 kb and gave 4,250 *inl*$^+$ *qa-2*$^+$ transformants per μg (double selection) and 3,750 *qa-2*$^+$ transformants per μg (single selection); 10 of 10 *qa-2*$^+$ transformants selected at random were found to be *inl*$^+$.

Mapping the chromosomal locations of the cloned DNA inserts. To confirm the identity of the putative clones, the chromosomal locations of the cloned DNA inserts were mapped by standard genetic crosses, using restriction site polymorphisms as genetic markers (16). Data from these crosses are shown in Fig. 4 and Tables 1 and 2. First, the putative *nic-1* clone (pRAL1-325532) was mapped by using the progeny of a standard cross obtained from the Fungal Genetics Stock Center. The parental strains in this cross were RLM1-33*a* (derived from Oak Ridge) which contains genetic markers *cot-1*, *al-2*, *arg-12*, *nuc-2*, and *inl*, and a natural isolate (Mauriceville-1c) which contains many restriction site polymorphisms when compared with the Oak Ridge strain. Figure 4A shows Southern blots in which pRAL1-325532, the putative *nic-1* clone, was hybridized to *Hin*cII digests of the parental strains and the standard set of progeny. The cloned insert in the plasmid hybridized to polymorphic fragments of 8.8 kb in the RLM1-33*a* (Oak Ridge; O) parent and 6.2 and 2.6 kb in the Mauriceville (M) parent and to a nonpolymorphic fragment of 1.7 kb in both strains. The hybridization band at 1.0 kb, which is observed in all strains, is due to the *N. crassa qa-2* gene present in the pRAL1 plasmid. (Additional bands in some of the lanes may reflect either incomplete digestions or cross-hybridization between the plasmid and *N. crassa* DNA fragments.) The hybridization patterns for the individual progeny show that the polymorphic fragments homologous to the cloned insert segregated with the chromosome IR marker, *al-2*, in 17 of 18 progeny, consistent with the distance of about 10 map units between *nic-1* and *al-2*. The polymorphism is linked to the mating type marker (linkage group IL) in these progeny but not to markers on other chromosomes (Table 1).

To confirm the identification of the *nic-1* clone, we used a mapping cross between Mauriceville-1c and another Oak Ridge-derived laboratory wild-type strain (RLM47-34*a*) which contains *nic-1* and other chromosome I markers. The progeny selected from this cross resolve markers on the left and right arms of linkage group I. The polymorphic *Hin*cII fragments segregate with the *nic-1* marker in all 16 progeny, as expected (Table 2).

The putative *inl* clone was mapped using progeny of the standard cross obtained from the Fungal Genetics Stock Center between RLM1-33*a* *cot-1*, *al-2* *nuc-2*, *arg-12*, *inl* and Mauriceville-1c (see above). Figure 4B shows a Southern blot in which pRAL1-724593, the putative *inl* clone, was hybridized to *Nru*I digests of DNAs of parental strains and the standard set of progeny. In this case, the putative *inl* clone hybridized to polymorphic restriction fragments that segregate with the *inl* marker in all of the progeny. The segregation of other markers in the cross is summarized in Table 1.

DISCUSSION

We have developed a sib selection procedure for cloning *N. crassa* nuclear genes by complementation of mutants. As a demonstration of this procedure, we describe here the cloning of the *N. crassa nic-1* and *inl* genes. More recently, we have used this procedure to clone additional genes by complementation of auxotrophic mutants, notably *his-2* (with John A. Kinsey, University of Kansas, Kansas City, Kans.) and *van* (isolated as a suppressor of *nuc-1*; with Barbara Mann and Robert L. Metzenberg; University of Wisconsin, Madison). We have also used the procedure to clone genes corresponding to temperature-sensitive mutants: *cyt(297-24)*, which encodes a protein required for assembly of mitochondrial small ribosomal subunits,

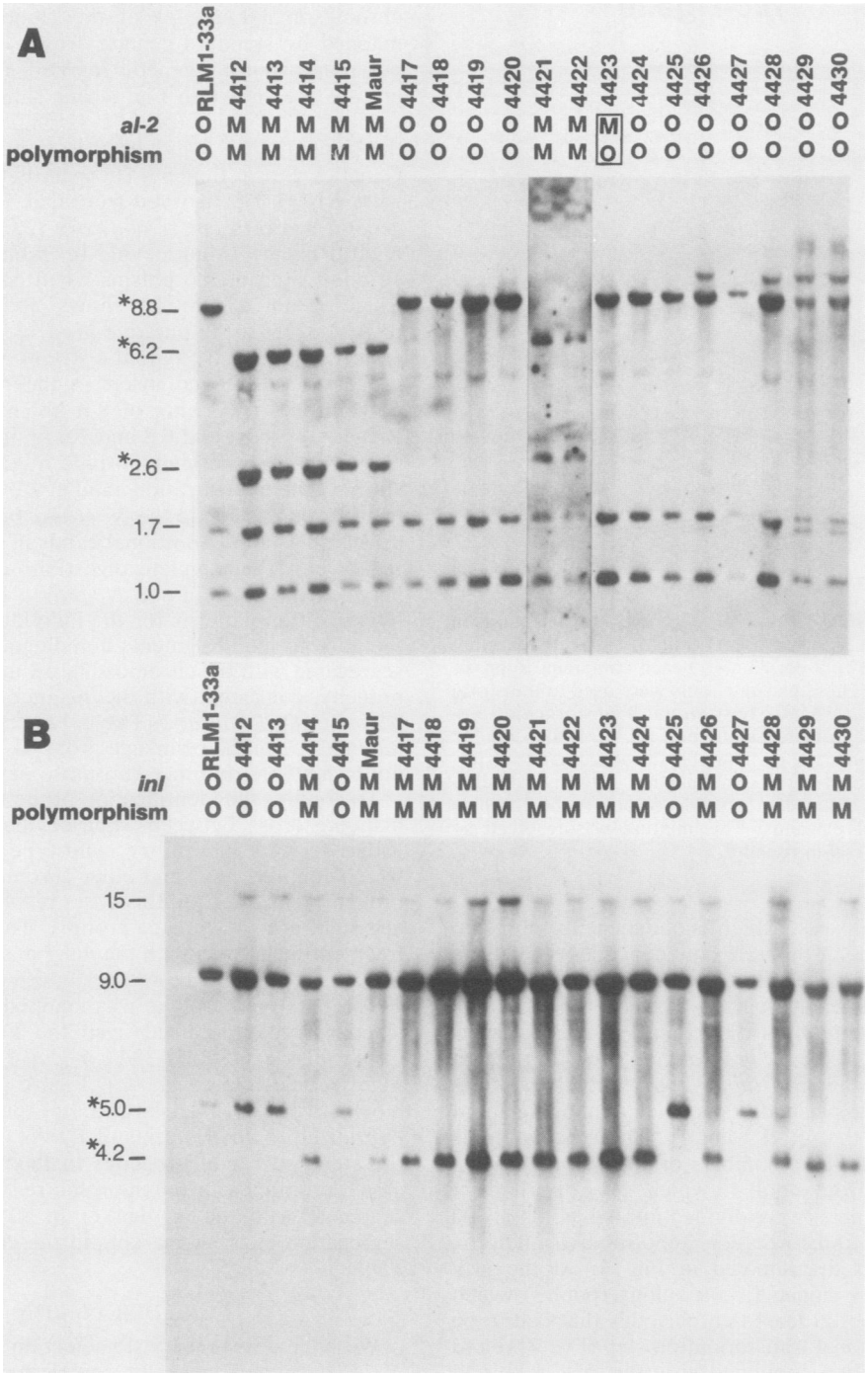

FIG. 4. Mapping of chromosomal locations of putative *nic-1* and *inl* clones in genetic crosses by using restriction site polymorphisms as genetic markers. Parental strains were RLM1-33*a cot-1 al-2 arg-12 nuc-2 inl* (derived from Oak Ridge; O) and Mauriceville-1c (M). Progeny are designated 4412 to 4430. Whole-cell DNAs from parental strains and selected progeny were digested with restriction enzymes, separated by electrophoresis in 0.8% agarose gels, and transferred to nitrocellulose. Blots were hybridized with ^{32}P-labeled recombinant plasmids containing putative *nic-1* or *inl* inserts. (A) Southern blot of *Hin*cII digests of parental strains and progeny hybridized with recombinant plasmid pRAL1-325532 which contains the putative *nic-1* insert. The cloned insert hybridizes to polymorphic fragments that are linked to the chromosome IR marker, *al-2*, in 17 of 18 progeny. Segregation of other markers in the cross is shown in Table 1. (B) Southern blot of *Nru*I digests of parental strains and progeny hybridized with pRAL1-724593 which contains the putative *inl* insert. The cloned insert hybridizes to polymorphic fragments that are linked to *inl* in 18 of 18 progeny. Segregation of other markers in the cross is shown in Table 1. Asterisks (*) indicate polymorphic restriction fragments that were used to determine Oak Ridge (O) or Mauriceville (M) genotypes of the progeny. *Hin*dIII-*Eco*RI fragments of phage lambda DNA were run in parallel lanes of the same gel as molecular weight standards (not shown).

TABLE 1. Segregation of genetic markers and restriction fragment polymorphisms in selected progeny of mapping cross RLM1-33*a* × Mauriceville-1c

Marker	Isolate no.:																			
	O[a]	4412	4413	4414	4415	M	4417	4418	4419	4420	4421	4422	4423	4424	4425	4426	4427	4428	4429	4430
nuc-2 (IIR)	O	O	O	M	M	M	M	M	O	O	M	O	M	O	O	M	O	O	M	M
arg-12 (IIR)	O	O	O	M	M	M	M	M	O	O	M	O	M	O	O	M	O	O	M	M
cot-1 (IVR)	O	M	M	M	M	M	M	M	M	M	O	O	O	O	O	O	O	O	O	O
mt (IL)	O	M	M	M	M	M	O	O	O	M	M	M	O	O	O	O	O	O	O	O
al-2 (IL)	O	M	M	M	M	M	O	O	O	O	M	M	M	O	O	O	O	O	O	O
pRAL1-325532[b]	O	M	M	M	M	M	O	O	O	O	M	M	O	O	O	O	O	O	O	O
inl (VR)	O	O	O	M	O	M	M	M	M	M	M	M	M	M	O	M	O	M	M	M
pRAL1-724593[c]	O	O	O	M	O	M	M	M	M	M	M	M	M	M	O	M	O	M	M	M

[a] Abbreviations: O, RLM1-33*a* (Oak Ridge); M, Mauriceville-1c.
[b] Putative *nic-1* clone.
[c] Putative *inl* clone.

(M. T. R. Kuiper, H. de Vries, R. A. Akins and A. M. Lambowitz, unpublished data) and *cyt-18*, which encodes a factor required for mitochondrial RNA splicing (unpublished data). At present, it seems likely that most *N. crassa* genes could be cloned by this method by using the pRAL1 library. Other types of libraries could also be used for sib selection cloning provided they give high *N. crassa* transformation frequencies.

To confirm the identification of the *nic-1* and *inl* clones, we mapped their chromosomal locations in standard genetic crosses, using restriction site polymorphisms as genetic markers (16). In both cases, the cloned inserts showed 100% linkage to the appropriate genetic marker in the crosses. These data provide very strong evidence that the correct clones have been obtained. We discount the formal but remote possibility that we have cloned closely linked suppressors of *nic-1* and *inl*.

There are several points which should be born in mind by other workers using the sib selection procedure. (i) The rapidity of the procedure described here is dependent upon modifications of the *N. crassa* transformation procedure, including the use of Novozym 234 to obtain high transformation frequencies and the use of frozen *N. crassa* spheroplasts and rapidly isolated *E. coli* plasmid DNAs to facilitate the multiple *N. crassa* transformation steps. (ii) The sib selection procedure could be carried out with lower transformation frequencies, either by scaling up the first transformation round or by starting directly with the second-round sib banks, each of which contains 300 plasmids. However, there are practical limits to the lowest transformation frequency that could be used. (iii) Plasmids such as pRAL1, which contain the *N. crassa qa-2* gene cloned in derivatives of pBR322, appear to be capable of some degree of autonomous replication in *N. crassa* (22, 23). At this point, we are not certain to what extent the high transformation frequencies obtained with pRAL1 reflect this ability. Thus, it is possible that transformation frequencies with other types of vectors or libraries will not be as high as those with pRAL1. (iv) It is necessary to establish tight selection to identify transformants. In our experience, the initial selection for double transformants (X^+ + *qa-2*$^+$, where *X* is the gene to be isolated) is mandatory for slow-growth mutants and facilitates the selection of auxotrophic mutants which are leaky or have high reversion frequencies. (v) A plasmid containing a specific cloned insert may be underrepresented in the *E. coli* banks at any stage in the sib selection for a variety of reasons, e.g., it may confer some selective disadvantage on *E. coli* transformants, or it may reduce the copy number of plasmids in which it resides. We have encountered this problem in cloning certain genes, but thus far it could always be overcome by constructing additional sib banks at the third or fourth round.

It is possbile that the sib selection procedure could be improved upon in the future by alternate approaches with plasmid vectors (22, 23) or cosmid vectors of the type recently developed for *Aspergillus nidulans* (28). In fact, we have obtained preliminary evidence that at least in some cases, genes can be isolated from transformants obtained with second-round sib banks by direct plasmid rescue (i.e., without ligation of transformant DNA). Although plasmid rescue is presently less reliable than sib selection, it may eventually provide a useful shortcut when transformants are difficult or tedious to select. In addition, rescue of plasmids from integrative transformants should be possible if one is willing to screen enough transformants to obtain integrants in which the transforming gene is flanked by plasmid se-

TABLE 2. Segregation of genetic markers and restriction fragment polymorphisms in selected progeny of mapping cross RLM47-34*a* × Mauriceville-1c

Marker	Isolate no.:																		
	4	6	12	13	16	17	20	21	23	25	26	27	28	29	30	35	M[a]	O
mt (IL)	M	M	O	O	O	M	M	O	O	M	M	O	M	O	M	M	M	O
nuc-1 (IR)	M	M	O	O	O	O	O	O	O	M	M	O	M	O	O	O	M	O
ad-9 (IR)	O	O	O	O	O	O	O	O	O	M	M	M	M	M	M	M	M	O
al-2 (IR)	O	O	O	O	O	O	M	M	M	O	M	M	M	M	M	M	M	O
nic-1 (IR)	O	O	O	O	O	O	M	M	M	O	M	M	M	M	M	M	M	O
pRAL1-325532[b]	O	O	O	O	O	O	M	M	M	O	M	M	M	M	M	M	M	O

[a] Abbreviations: O, RLM47-34*a* (Oak Ridge); M, Mauriceville-1c.
[b] Putative *nic-1* clone.

quences. Although the development of these alternate approaches should be pursued, our experience has been that the most time-consuming steps in the gene cloning are establishing appropriate selection conditions and verifying that bona fide transformants have been obtained. Once these steps have been accomplished, the sib selection procedure can be completed almost as rapidly, if not as rapidly, as any of the direct rescue procedures.

The sib selection procedure described here is the first shown to be generally useful for cloning *N. crassa* nuclear genes by complementation of mutants. The procedure can be used now to clone any *N. crassa* nuclear gene that is the site of a mutation for which a selection can be devised. Because of the high *N. crassa* transformation frequencies that we can obtain, cloning can be accomplished with as few as five subdivisions of an *N. crassa* genomic library (4 to 8 weeks), even in the absence of further improvements. Analogous procedures may be generally applicable to other filamentous fungi before the development of operational shuttle vectors.

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ADDENDUM IN PROOF

We have found that osmolarity is also an important parameter for obtaining high *N. crassa* transformation frequencies with Novozym 234. The highest transformation frequencies were obtained by diluting 100 μl of spheroplasts in transformation buffer with 60 μl of distilled water at the time DNA was added.

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