Inactivation of the *Neurospora crassa* Gene Encoding the Mitochondrial Protein Import Receptor MOM19 by the Technique of "Sheltered RIP"

Troy A. A. Harkness,* Robert L. Metzenberg,† Helmut Schneider,‡ Roland Lill‡, Walter Neupert‡ and Frank E. Nargang*

**Department of Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2E9, †Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706-1532 and ‡Institut für Physiologische Chemie, Universität München, 80336 München, Federal Republic of Germany.*

Manuscript received July 20, 1993
Accepted for publication October 4, 1993

ABSTRACT

We have used a technique referred to as "sheltered RIP" (repeat induced point mutation) to create mutants of the *mom-19* gene of *Neurospora crassa*, which encodes an import receptor for nuclear encoded mitochondrial precursor proteins. Sheltered RIP permits the isolation of a mutant gene in one nucleus, even if that gene is essential for the survival of the organism, by sheltering the nucleus carrying the mutant gene in a heterokaryon with an unaffected nucleus. Furthermore, the nucleus harboring the RIPed gene contains a selectable marker so that it is possible to shift nuclear ratios in the heterokaryons to a state in which the nucleus containing the RIPed gene predominates in cultures grown under selective conditions. This results in a condition where the target gene product should be present at very suboptimal levels and allows the study of the mutant phenotype. One allele of *mom-19* generated by this method contains 44 transitions resulting in 18 amino acid substitutions. When the heterokaryon containing this allele was grown under conditions favoring the RIPed nucleus, no MOM19 protein was detectable in the mitochondria of the strain. Homokaryotic strains containing the RIPed allele exhibit a complex and extremely slow growth phenotype suggesting that the product of the *mom-19* gene is important in *N. crassa*.

THE filamentous fungus, *Neurospora crassa*, has proven to be an excellent organism for many biochemical investigations. The ease by which biochemical questions can be addressed has been contrasted, until very recently, by the relative difficulty of isolating mutants in specific target genes. In the yeast *Saccharomyces cerevisiae*, there are various methods for creating null alleles of genes that have been cloned. Perhaps the most common is by integrative replacement of the endogenous gene with a copy of the gene that has been interrupted by a large DNA sequence, usually a selectable marker (ROTHSTEIN 1991). Limited success using this approach has also been achieved with *N. crassa* (*e.g.*, FREDERICK, ASCH and KINSEY 1989; NEHLS *et al.* 1992), but the frequency of replacement by homologous integration may be very low for many *N. crassa* genes (ASCH and KINSEY 1990). An efficient alternative to methods of disruption based on homologous replacement is to utilize the RIP (repeat induced point mutation) phenomenon that has been described in *N. crassa* (SELKER 1990). The process of RIP affects duplicated DNA sequences present in a nucleus that participates in a genetic cross and effectively results in the disruption of both copies of the duplication by the generation of numerous GC to AT transitions. Thus, a strain engineered by genetic transformation to contain a duplication of any *N. crassa* gene could be used to generate specifically mutants in that gene. This approach has already been used to create mutants in various genes in *N. crassa* (*e.g.*, FINCHAM *et al.* 1989; GLASS and LEE 1992). Thus, gene RIP in *N. crassa* and gene replacement procedures in yeast both result in the isolation of strains with nonfunctional target genes.

However, for genes providing important functions to the cell, the rather simple approach described above would not permit the isolation of mutants since they might be inviable and/or have severely reduced germination rates. If such a gene was essential for growth or germination of ascospores, the RIP method should produce genetic data indicating that this was the case. That is, a certain percentage of tetrads, related to the frequency of RIP of the target sequence, should produce only four viable spores instead of the usual eight. The latter situation is analogous to sporulation of a diploid yeast carrying a disruption in one nucleus where failure to isolate the relevant haploid strain indicates that the target gene is essential for germination and/or growth (ROTHSTEIN 1991). In both organisms, the genetic data are useful but strains with the mutant phenotype, which might be utilized for further study, are not produced because they are inviable. In yeast, one way to overcome this problem is by supplying an extra copy of the target gene, under the control of a promoter that is induced by galactose and is repressed by glucose, to the disruptant-contain-

ing nucleus prior to meiosis. Therefore, in the presence of galactose and the absence of glucose, the disruptants will germinate and grow since the spores are supplied with the essential gene product (BAKER *et al.* 1990; SCHNEIDER and GUARENTE 1991). However, when such a culture is shifted to medium lacking galactose and containing glucose, the gene product is gradually diluted out of the cells so that the effect of the deficiency can be monitored in the cells at various stages of depletion of the product. Unfortunately, there are no promoters with a suitable degree of control currently available for performing similar experiments in *N. crassa*. Therefore, an extension of the RIP procedure, which we refer to as "sheltered RIP," has been developed to achieve a similar outcome (METZENBERG and GROTELUESCHEN 1992a). This technique allows the isolation of a mutant gene in one nucleus, even if that gene is essential for the survival of the organism, by sheltering the nucleus carrying the mutant gene in a heterokaryon with an unaffected nucleus. Furthermore, the inclusion of a selectable marker in the nucleus harboring the RIPed gene makes it possible, by growing cultures under the appropriate conditions, to shift the nuclear ratios in the heterokaryons at will, to a state in which the nucleus containing the RIPed gene predominates. This gives rise to a condition in which the product of the gene that has been RIPed is present at very suboptimal levels and allows the study of the mutant phenotype. The latter situation should be analogous to the conditions created when the yeast disruptant is shifted to glucose medium lacking galactose. In both cases, alterations in the composition of the medium can be used to "control" the amount of the essential product present in the culture.

In this report, we describe the successful application of sheltered RIP for generating mutations in the *mom-19* gene. The product of the gene, MOM19, is a protease-sensitive surface component of the protein import complex of the mitochondrial outer membrane (SÖLLNER *et al.* 1989). Biochemical studies, using MOM19 specific antibodies, have demonstrated that MOM19 serves as the initial receptor site for the import of the vast majority of nuclear-encoded mitochondrial preproteins (SÖLLNER 1989). A second receptor, MOM72, was found to play a more specialized role in the import of the ATP/ADP carrier (SÖLLNER *et al.* 1990). Following interaction with MOM19 or MOM72, the preproteins are transferred to a general insertion pore called GIP (PFALLER *et al.* 1988). A major component of GIP is MOM38 (KIEBLER *et al.* 1990) or its yeast homologue, ISP42 (BAKER *et al.* 1990). Transfer of the preproteins from the receptor stage to the GIP stage is facilitated by a membrane-spanning component of the receptor complex called MOM22 (KIEBLER *et al.* 1993).

Since MOM19 plays such a pivotal role in the import of the majority of proteins into mitochondria, we suspected that inactivation of the gene encoding the protein might have severe consequences on *N. crassa* cells. For this reason, we have used sheltered RIP to isolate mutants in this gene. Here we describe both the genetic details of the procedure as applied to *mom-19* and the characteristics of a mutant strain that is grossly deficient in the MOM19 protein when grown under appropriate conditions.

MATERIALS AND METHODS

Strains and media: Growth and handling of *N. crassa* strains was as described in DAVIS and DE SERRES (1970). All strains used in this study are listed in Table 1. The Host IV and Mate IV strains carry complementary nutritional markers on linkage group IV (LG IV) that allow for specific selection of LG IV disomic ascospores following crosses of Mate IV and transformed derivatives of Host IV (see RESULTS). LG IV disomics are the only spores generated from the cross that are capable of growth on basal media lacking tryptophan (*trp-4* marker) and uridine (*pyr-1* marker). Thus, we define basal medium as the medium capable of supporting growth of disomics or heterokaryons containing both the Host and Mate LGs IV. The medium contains Vogel's salts including trace elements and biotin (DAVIS and DE SERRES 1970), 1.5% glucose and inositol (50 μg/ml). When required, uridine (1 mM) and tryptophan (0.5 mM) were added to the medium. *p*-fluorophenylalanine (fpa) was used at various concentrations as given in the text. Though not required by the strains used in this study, either leucine (1 mM) or arginine (1 mM) were also present in media because they are part of a standard medium used in our laboratories for similar experiments.

Plasmid construction: Both genomic and cDNA versions of the *mom-19* gene were cloned previously (SCHNEIDER *et al.* 1991). We utilized the genomic clone to construct a plasmid, pKSH6 (Figure 1) that is a derivative of pBR322 containing the *mom-19* sequence and a bacterial *hph* gene (hygromycin B phosphotransferase) expressed by a *trpC* promoter from *Aspergillus nidulans* (CULLEN *et al.* 1987). Resistance to hygromycin allows selection of transformants in *N. crassa* (STABEN *et al.* 1989). The *N. crassa* DNA in pKSH6 totals 1607 bp and extends only 39 bp upstream of the *mom-19* cDNA start site and 108 bp downstream of the cDNA end point. In experiments designed to generate RIP mutations in specific target genes, it is desirable to limit the amount of non-*mom-19* DNA in the construct to prevent RIP of neighboring genes.

Isolation of mitochondria: Mycelium from liquid cultures was harvested by filtration. The mycelium was weighed and ground by hand using a mortar and pestle in the presence of 1.5 g of acid-washed quartz sand and 1 ml of grinding buffer (0.44 M sucrose; 10 mM Tris-HCl, pH 7.5; 0.2 mM ethylenediaminetetraacetic acid; 0.2 mM phenylmethylsulfonyl fluoride) per gram of mycelium. When a smooth paste was formed, the slurry was centrifuged at 3,000 rpm in a Sorvall SS-34 rotor for 10 min to remove nuclei, cell debris and sand. The supernatant was transferred to a clean tube and centrifuged for 20 min at 12,000 rpm in the same rotor. The supernatant was discarded and the mitochondrial pellet was washed once in grinding buffer. The final pellet was suspended in a small volume of grinding buffer and stored at −80°.

Analysis of genomic DNA for evidence of RIP: Restriction digests of genomic DNA with enzymes chosen to detect RIP were electrophoresed on 5% polyacrylamide gels (29:1,

TABLE 1

Strains used

Strain	Genotype or origin
Host IV[a]	LG I, *a*; LG IV, *pyr-1 mom-19*$^+$ *mtrR trp-4*$^+$; LG V, *inl inv mei-2*.
Mate IV[b]	LG I, *A*; LG IV, *pyr-1*$^+$ *mom-19*$^+$ *mtrS trp-4*; LG V, *am inl inv mei-2*.
TI26-3	Isolate from transformation of Host IV with pKSH6, contains single ectopic copy of *mom-19*, hygromycin resistant.
TI28-3	As TI26-3.
TI40-3	As TI26-3.
28.1	Ascospore isolate of Mate IV × TI28-3 cross, selected as heterokaryon on basal medium, status of *mom-19* gene unknown.
28.16	As 28.1, except that one component of the heterokaryon, containing the LG IV derived from the Host IV strain, is known to contain a RIPed allele of *mom-19*. Both components of the heterokaryon contain an ectopic RIPed version of the mom-19 sequence derived from integration of pKSH6.
28.17	As 28.16.
28.18	As 28.1.
28.23	As 28.1.
M17-60	Homokaryon isolated from 28.17 containing RIPed allele of *mom-19*.
M17-63	As M17-60.
M17-65	As M17-60.
M17-67	As M17-60.
M17-69	As M17-60.
M17-70	As M17-60.
M17-71	As M17-60.
M17-72	As M17-60.
M17-73	As M17-60.
M17-74	As M17-60.
M17-75	As M17-60.
M170-76	As M17-60.
17-43.1	Transformant of strain 28.17 obtained by transformation with a cosmid containing the *mom-19* gene and approximately 35 kb of additional *N. crassa* genomic DNA. Isolated on the basis of increased growth rate on basal medium plates containing sorbose and supplemented with 600 μM fpa plus uridine. Shown to be homokaryon containing LG IV from Host IV. MOM19 protein present.
17-67.1	As 17-43.1 except that transformation was with a plasmid containing the *mom-19* gene and an additional 4 kb of *N. crassa* genomic DNA.
17-94.2	As 17-43.1 except that transformation was with a plasmid containing mom-19 cDNA.

[a] The Host IV strain used in these experiments is carried as an alternate Host IV at the Fungal Genetics Stock Center (strain #7270), the standard Host IV strain (Fungal Genetics Stock Center strain #7254) is *pan-1* instead of *pyr-1*.

[b] This strain is used as a heterokaryon with an inactive mating type strain (allele a^{m1}) carrying an *ad-3B* marker so that nutritional complementation is possible with the Mate IV nucleus. Thus, the heterokaryotic strain can be grown as a prototroph on minimal medium to increase fertility in genetic crosses. When this heterokaryon is used in a cross, only the Mate IV nucleus, which has an active mating type allele, is carried through the cross (GRIFFITHS and DELANGE 1978; GRIFFITHS 1982; PERKINS 1984).

acrylamide:bisacrylamide) in 1× TAE buffer (40 mM Tris-acetate, pH 8.3; 1 mM EDTA) and electroblotted to nylon membranes using 0.5× TAE as the blotting buffer. The membrane was then placed on 3MM chromotography paper (Whatman) saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10 min. Following denaturation, the membrane was neutralized by placing it on 3MM paper saturated with 3 M sodium acetate, pH 5.5 for 10 min. The membrane was then air dried and baked at 80° for 1 hr. Hybridization of the membrane was by standard techniques (AUSUBEL *et al.* 1992) except that the hybridization and wash temperatures were reduced from 65° to 57°, since the size of many of the restriction fragments was predicted to be small.

Other techniques: The standard techniques of agarose gel electrophoresis and Southern blotting of agarose gels, preparation of probes for DNA-DNA hybridizations, transformation of *Escherichia coli*, isolation of bacterial plasmid DNA and the polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs, Beverly, Mass.) to minimize replication errors were performed as described in AUSUBEL *et al.* (1992). The following procedures were also performed using previously published procedures: separation of mitochondrial proteins by polyacrylamide gel electrophoresis (LAEMMLI 1970), Western blotting (GOOD and CROSBY 1989), *N. crassa* DNA isolation (SCHECHTMAN 1986), determination of mitochondrial protein concentration (BRADFORD 1976), preparation of antibodies to mitochondrial proteins (SÖLLNER *et al.* 1989), transformation of *N. crassa* spheroplasts (SCHWEIZER *et al.* 1981) with the modifications of AKINS and LAMBOWITZ (1985), and restriction fragment length polymorphism (RFLP) analysis of *N. crassa* genes (METZENBERG *et al.* 1984, 1985). DNA sequences were obtained using Sequenase (United States Biochemical) according to the supplier's instructions. Database information with regard to the sequences obtained is given in the legend to Figure 5.

RESULTS

Principles of "sheltered RIP": Because we suspected that loss of MOM19 would have an extremely deleterious effect on the organism, we utilized a procedure referred to as "sheltered RIP" to generate mutants of the *mom-19* gene. The method utilizes the phenomenon of RIP (SELKER 1990), to destroy the target gene. When a DNA sequence is present in two

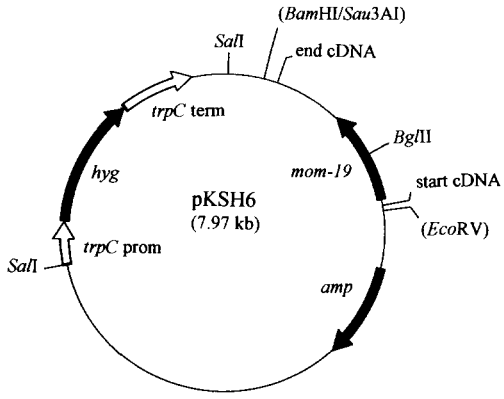

FIGURE 1.—Plasmid pKSH6. Names of genes are indicated inside the circle. Filled arrows indicate the extent of coding sequences, open arrows indicate the promoter and terminator regions derived from *Aspergillus nidulans*. Restriction sites are indicated outside the circle. The sites in parentheses indicate the cloning sites and extent of the *mom-19* genomic sequence in the plasmid. The positions that correspond to the start and endpoints of the *mom-19* cDNA sequence are also indicated to demonstrate that the genomic DNA in the plasmid does not extend significantly past *mom-19* sequence.

copies in a single nucleus both copies are, with fairly high frequency, subjected to RIP in the ascogenous hyphae as the nucleus is prepared for karyogamy. Thus, any gene that has been cloned can be reintroduced into a strain by transformation to create a duplication in which both copies serve as targets for RIP. Most frequently the reintroduced gene integrates at an ectopic site (ASCH and KINSEY 1990). The result of RIP is that both copies of the sequence in the transformed nucleus are effectively disrupted since they are altered by methylation and by GC to AT transitions. The single copy of the gene in the nucleus of the mating partner in the cross is unaffected by RIP. To allow the isolation of strains containing essential genes that have been RIPed during the cross, the technique employs a mutant allele of the *mei-2* gene in both partners of the cross. This mutation effectively eliminates meiotic recombination and also results in a high frequency of nondisjunction of chromosomes during meiosis (SMITH 1975). Thus, in crosses homozygous for *mei-2*, a variety of meiotic products are generated, many of them inviable because they are nullosomic for one or more chromosomes. However, a fraction of the meiotic products should be disomic for any specific chromosome. The desired products of a sheltered RIP cross will be disomic ascospores containing a RIP disruption of the target gene on one copy of a certain chromosome (derived from the transformation host) and a normal copy of the target gene on another copy of the same chromosome (derived from the mating partner of the cross). These disomic spores break down rapidly during vegetative growth into a heterokaryon composed of two different nuclei (SMITH 1974). Even if the target gene is essential for viability, the normal copy of the gene in one component of the heterokaryon will complement, or shelter, the nonfunctional copy in the other component. Knowledge of the linkage group on which the target gene is located and appropriate genetic design of the strains used in the cross allows the direct selection of the desired dikaryons/heterokaryons from the progeny of the cross, as well as providing selectable markers that can be used to alter the ratio of the different nuclei in the ultimate heterokaryon (see below). Therefore, it is possible to produce a strain in which the nucleus containing the disrupted version of the gene should be heavily favored numerically, though if the target gene provides an indispensible function it can never become the sole nuclear type. These skewed-ratio heterokaryons should allow the growth-limited phenotype to be studied. In principle, the technique should be useful for generating mutants of any cloned gene in Neurospora though it is particularly well-suited for genes that have an important or essential function (METZENBERG and GROTELUESCHEN 1992a). Below we describe the details of the technique specifically as applied to the *mom-19* gene.

Transformants containing *mom-19* duplications: The principle of sheltered RIP, which employs various genetic markers on the chromosome carrying the target gene, requires that the chromosome carrying the target gene locus be known. Therefore, *mom-19* specific sequences were used as a probe to isolate *mom-19* containing cosmids from a *N. crassa* library. One cosmid was then utilized as a probe to deduce the chromosomal location of the *mom-19* locus in RFLP mapping studies (not shown). The analysis revealed that *mom-19* is located on LG IV as shown in METZENBERG and GROTELUESCHEN (1992b). Plasmid pKSH6, which carries the *mom-19* gene (see MATERIALS AND METHODS), was used to transform the Host IV strain. This strain carries a number of relevant markers: the *pyr-1* and *trp-4*$^+$ alleles to be utilized in the selection of appropriate disomic ascospores following the eventual RIP cross, a recessive LG IV mutation imparting resistance to fpa or 5-methyltryptophan (*mtrR*) which should allow shifts in nuclear proportions favoring the RIPed nucleus in the isolates ultimately obtained from the RIP cross, and a mutant allele of *mei-2* to generate disomics in the RIP cross. The genetic makeup of appropriate transformants is shown in Figure 2A.

Transformants were selected on media containing hygromycin. Sixty transformants were purified through two rounds of single colony isolation on hygromycin medium to ensure that the transformants were homokaryotic. DNA was isolated from 35 of these transformants and examined for the presence of a single inserted copy of the *mom-19* sequences derived from pKSH6, in addition to the resident copy of *mom-19*. This was tested by using a *mom-19* specific sequence to probe Southern blots of genomic DNA

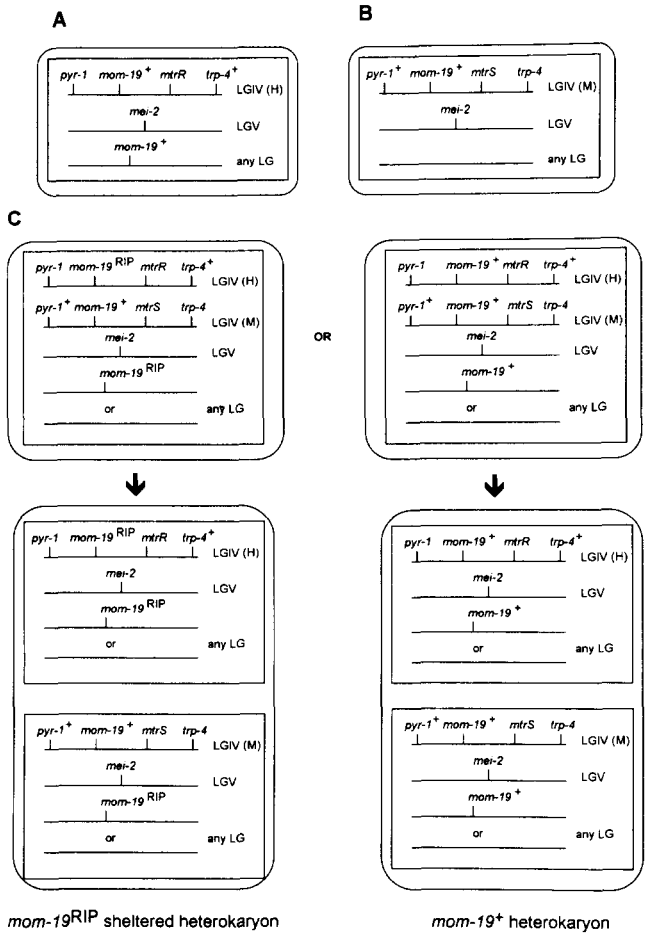

FIGURE 2.—Relevant genetic markers in appropriate initial transformants, the Mate IV strain, disomic progeny isolates and subsequent heterokaryons. Only the genetic markers that are important for generation, selection or manipulation of the strains are shown. These are indicated on specific linkage groups represented by horizontal lines. The position of *mom-19* relative to the other markers on LG IV is shown as deduced from both the RFLP map (METZENBERG and GROTELEUSCHEN 1992b) and the genetic map of LG IV (PERKINS 1992). LGIV (H), linkage group IV derived from the original Host IV strain; LGIV (M), linkage group IV derived from the original Mate IV strain; LGV, linkage group V; any LG, any of the seven linkage groups found in *N. crassa*. Boxes with squared corners indicate nuclei, boxes with rounded corners indicate cells. (A) Desired initial transformants of the Host IV strain containing a single ectopic copy of *mom-19*. (B) The Mate IV strain. (C) The top two cells indicate the two possible versions of disomic spores that would grow on media lacking both uridine and tryptophan. Each must contain one LG IV from the original host strain and one LG IV from the original mate strain to allow complementation of the auxotrophic *pyr-1* and *trp-4* markers. One type contains two unaltered copies of *mom-19*; the other contains one unaltered copy and one RIPed copy. In both cases the ectopic copy of *mom-19* may or may not be present depending on the meiotic segregation of the chromosome on which it was integrated. Each of the disomics breaks down into a heterokaryon in which one nucleus contains LG IV from the host and the other LG IV from the mate as shown in the bottom two cells of part C of the figure.

isolated from the transformants and cut with either *Bgl*II or *Eco*RV plus *Sal*I. *Bgl*II cuts within *mom-19* DNA and, in appropriate transformants containing a single ectopically integrated *mom-19* sequence, should generate four bands when probed with *mom-19* specific DNA: two bands from the ectopic copy and two from the resident copy. The size of the bands from the ectopic copy cannot be predicted and depends on the position of flanking *Bgl*II sites. Appropriate strains should contain bands that correspond to those in the parental strain Host IV, and two additional bands. Both *Eco*RV and *Sal*I cut outside the *mom-19* coding sequence and outside the *mom-19* DNA present in pKSH6 (Figure 1). Therefore, the double digest should generate two bands in appropriate transformants. One band should be 1.8 kb, representing the ectopic sequence derived from integration of the *mom-19* sequence in pKSH6. The other band represents the resident *mom-19* sequence and should correspond to the band in the Host IV strain. Three strains that satisfied these criteria were identified from the analysis: TI26-3, TI28-3 and TI40-3 (Figure 3). Each of these strains was used as the male parent in crosses to strain Mate IV, which carries the *mei-2* allele on LG V as well as nutritional markers on LG IV that are complementary to those on LG IV of the transformed strains (Figure 2B). In the present study we have concentrated on analysis of the progeny from the cross involving TI28-3.

Generation and characterization of *mom-19* RIP mutants: Crosses between the Mate IV strain and transformant TI28-3 are homozygous for the *mei-2* allele so that during meiosis nondisjunction of chromosomes occurs and meiotic recombination is inhibited. Therefore, a fraction of the ascospores obtained from the cross should be disomics containing one LG IV derived from the Mate IV strain and one derived from the Host IV transformed strain (Figure 2C). Such ascospores can be directly selected because they are the only ones produced from the cross that are capable of growth on medium that lacks both uridine (*pyr-1* marker) and tryptophan (*trp-4* marker). When the cross is carried out, a certain percentage of the nuclei carrying the duplication of *mom-19* should undergo RIP of those sequences. Therefore, two types of disomics should emerge from the selection. The first type would contain one LG IV derived from the Host IV transformed strain with a RIPed version of the gene and one LG IV derived from the Mate IV strain carrying a wild-type *mom-19* allele. The second type would contain two wild-type alleles of *mom-19* (Figure 2C). The spores of the first type are the desired isolates and their frequency will be related to the frequency of RIP of the *mom-19* duplication in the original transformed host. Depending on the site of integration of the transformed ectopic *mom-19* gene and the pattern of segregation of chromosomes during meiosis, the spores may or may not contain the ectopic integrated copy of *mom-19* as well ("any LG," Figure 2). If the ectopic copy is present in the spores that contain resident *mom-19* sequences that have been subjected to RIP, the ectopic sequence should also

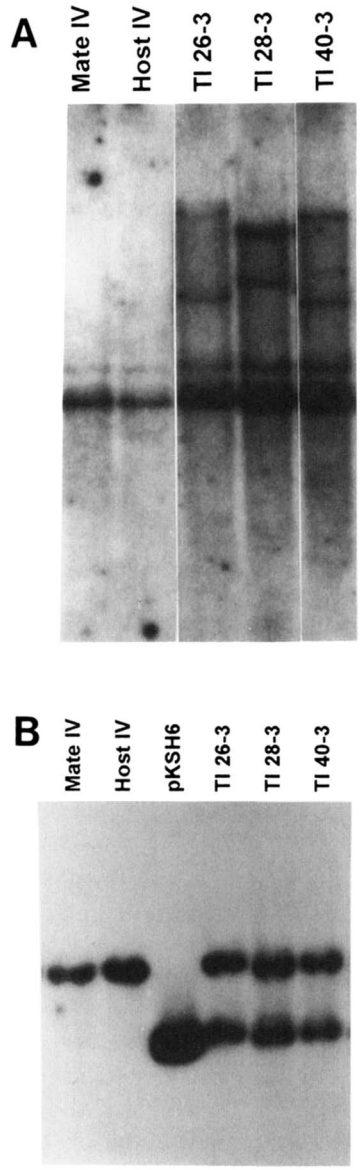

FIGURE 3.—Southern analysis of restriction digests showing single inserted *mom-19* sequence in strains TI26-3, TI28-3, TI40-3. (A) *Bgl*II digest of total cellular DNA from the indicated strains. (B) *Eco*RV plus *Sal*I digest of total cellular DNA from the indicated strains. Also shown is the 1.8-kb marker generated from a digest of plasmid pKSH6. The probe was a radioactively labeled *mom-19* cDNA fragment that extends from nucleotides 245–1704 as shown in Figure 5, except that the intron sequences shown in the figure are absent in the cDNA.

have undergone RIP since both copies of a duplication are affected by the process (SELKER 1990).

Ascospores produced from the Mate IV × TI28-3 cross were plated on basal medium (see MATERIALS AND METHODS), which lacks uridine and tryptophan. A total of 50 colonies that formed on this medium were picked to culture tubes containing basal medium and allowed to conidiate. Each strain was then taken through two rounds of purification by streaking to single colony isolates on basal medium to provide sufficient opportunity for breakdown of disomic nuclei (SMITH 1974) and to maintain the heterokaryons formed during the process of breakdown. The isolates were then examined to determine which, if any, carried RIPs of *mom-19* in the *pyr-1 trp-4*$^{+}$ *mtrR* component of the heterokaryon. We tested for such strains by examining the growth properties of each isolate on medium that is both selective and permissive for the RIPed nucleus, that is, containing uridine and fpa but not tryptophan. The heterokaryotic strains isolated should all carry the recessive resistance marker *mtrR* and should therefore be capable of growth on this medium by spontaneous resolution to give *mtrR* homokaryons. However, if the nucleus carrying *mtrR* contains a RIPed copy of *mom-19*, and *mom-19* is required to maintain a normal rate of growth, then such strains should not give rise to vigorously growing *mtrR* homokaryons. In such cases, the heterokaryons should exhibit a reduced growth rate at some critical concentration of the inhibitor, since the nucleus carrying the RIPed *mom-19* allele is forced to predominate in the heterokaryon. In Table 2 we show the results obtained with five candidate strains that exhibited altered growth patterns on the inhibitor relative to control strains.

As a test for RIP of *mom-19* that was not influenced by any prediction with regard to the functional importance of the gene, we examined the *mom-19* DNA in the strains discussed in Table 2 for evidence of restriction site alterations that should be produced by the RIP process. DNA was isolated from cultures of each of these strains, digested with restriction enzymes and compared with digests of the parent strains used in the RIP cross by Southern analysis using a *mom-19* specific sequence as the probe. The enzyme combinations chosen enabled us to determine if alterations were simply due to methylation or to actual base pair changes. The enzymes *Sau*3AI and *Mbo*I both cut the sequence GATC. However, *Sau*3AI will not cut if the C is methylated and *Mbo*I will not cut if the A is methylated. Virtually all methylation in Neurospora is at cytosine residues so that digestion with *Mbo*I should not be affected by methylation. *Hpa*II and *Msp*I both cut the sequence CCGG. *Hpa*II will not cut if either C is methylated. *Msp*I will not cut if the outer C is methylated. The enzymes were used in the combinations *Sau*3AI plus *Hpa* II and *Mbo*I plus *Msp*I to generate many restriction fragments, thereby maximizing the chances of detecting alterations due to methylation or restriction site alterations. As shown in Figure 4, it is apparent that strains 28.16 and 28.17 have alterations with respect to control DNAs. For strain 28.16, the alterations may be explained by differences in methylation alone since there are no apparent differences from controls in the *Msp*I plus *Mbo*I digest. However, for 28.17, the alterations are likely the result of both methylation and mutation. For both strains we have shown that the alterations are specific for *mom-19* DNA by stripping the blots

TABLE 2

Growth of heterokaryons on medium containing *p*-flurophenylalanine

Strain	Hours required to cover surface of slant[a] containing *p*-fluorophenylalanine at indicated concentration							
	0 μM	100 μM	200 μM	300 μM	400 μM	500 μM	600 μM	700 μM
TI28-3	24	24	24	24	24	24	24	48
Host IV	24	24	24	24	24	24	24	48
Mate IV	24–48	>96	No gr[b]	No gr	No gr	No gr	No gr	No gr
28.1	24–48	24–48	72	96	96	96	96	96
28.16	24–48	24–48	48	Lost	48	48	48	48
28.17	24–48	24–48	72	72	72	72	72	72
28.18	24–48	24–48	>96	>96	>96	>96	>96	>96
28.23	24–48	24–48	48	48	48	48	48	48

[a] Slants were prepared in 16 × 150 mm tubes. Average length of the slant was about 80 mm. Conidia were inoculated at a single point in the center of the slant.

[b] No gr, no growth after 96 hr.

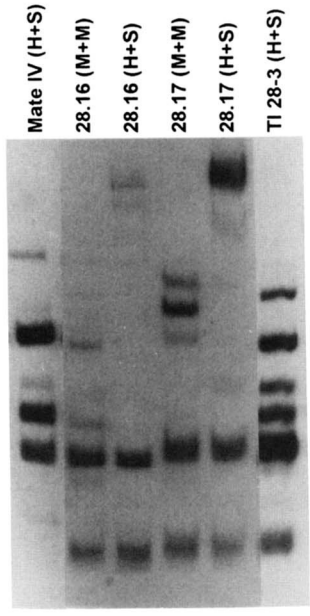

FIGURE 4.—Southern analysis of restriction digests showing RIP in strains 28.16 and 28.17. DNA isolated from the indicated strains was digested with the restriction enzymes indicated. (H+S) = *Hpa*II+*Sau*3AI; (M+M) = *Msp*I+*Mbo*II. The probe was a radioactively labeled *mom-19* cDNA fragment as described in the legend to Figure 3.

and hybridizing with a different probe. In this case, no differences in banding patterns were seen in any strain. Although no alterations in *mom-19* were apparent in strains 28.1, 28.18 and 28.23 (not shown), it is conceivable that changes have occurred that are not detected by the restriction enzymes used in the analysis.

DNA sequence analysis of *mom-19*^{RIP} alleles: To fully characterize the extent of RIP that had occurred in strains 28.16 and 28.17, we isolated *mom-19* DNA from these strains by PCR on DNA isolated from cultures grown under conditions that force the maintenance of the heterokaryon (basal medium). Since we had demonstrated by Southern analysis that both strains carried the ectopic, or plasmid derived copy of *mom-19* in addition to the resident copies on the two LGs IV (not shown), two sets of PCR primers were used in these experiments. One set was designed to amplify the introduced copy of *mom-19* carried on plasmid pKSH6 and the other set was designed to amplify the resident genomic *mom-19* sequence. The products of each reaction were cloned into bacterial vectors and their DNA sequences were determined. As expected, the products of the PCR reaction using the primers for the resident sequence gave rise to two types of clones. One type was of the wild-type *mom-19* sequence derived from LG IV of the Mate IV strain. The other type gave DNA sequence with alterations from the wild-type sequence. The latter type represents the RIPed version of the gene present on the LG IV originally derived from the transformed Host IV strain. For strain 28.17 a total of 44 changes from the wild-type sequence were observed in the 1134 base pairs of duplicated sequence examined (Figure 5). All these alterations were of the type that characterize the RIP process, GC base pair to AT base pair transitions. The strand bias of mutation that is typical of RIP is also evident (CAMBARERI *et al.* 1989). The mutations are predicted to result in a total of 18 amino acid substitutions in the MOM19 protein (Figure 5). Most of the amino acid changes are conservative but one, at nucleotide position 671, results in a Glu to Lys substitution. The ectopically integrated copy of *mom-19* in strain 28-17 has not been sequenced entirely; but from 870 bp completed thus far, we have found 32 transitions that would result in 12 amino acid substitutions. It is extremely doubtful that any functional *mom-19* is produced from this ectopic *mom-19* gene since the 5′ splice junction of the third intron is also affected by one of the alterations. We have not completed the sequence of the RIPed alleles from strain 28.16 but preliminary analysis indicates several transitions characteristic of RIP in this strain as well.

MOM19 protein in mutant strains: To investigate further the status of the *mom-19* gene in the strains that showed evidence of RIP on Southern blots, we examined strains 28.16 and 28.17 for the presence of

```
AAAAACACAGTTTGTGTAGTGTACGGGTACGATAATTACGGTATGTGCAATGTTTTGACCTGCAAGCTTACCTCTCCTCTAACCATCCAATCTTTGAGAGTGCTGTTGATGTCCATTGCT  120

TGTGAACCTTCCCGTGGAGCCAGTGTCATATACTGACCCCCACTTGCAGCAGTCTTTCCGGTAGTACTAAGAAATCGAGAGGCTCGATATCCATCGCATCTTTCCCTACCATTATAATTT  240

                    A                                                                                   A
CTCTCTCTCGACTCTCCCCCGCATCCACCATCACCACACAACGAGCCAACAAGATGCCGTCGCAAGCCGTCACCTACACCACCGCTGCCGTTGCGGCCGTCGCCACGGGTTTCCTCGgtt  360
                                                MetProSerGlnAlaValThrTyrThrThrAlaAlaValAlaAlaValAlaThrGlyPheLeu
                                                                                                    Thr

                                                          a       a                                 A       A
agtttgcacttggatctcaacgtcaccactttctcgagatgtaaccattagtcgctgatgccttgcgctccctaccttccagCCTACGCCGTCTACTTCGACTACAAGAGGCGCAATGA  480
                                                                                                AlaTyrAlaValTyrPheAspTyrLysArgArgAsnAsp
                                                                                                    Ile     Asn

                          A
CCCCGAGTTCAGGAGACAACTCAGGCGCAGCGCCAGGCGACAGGCTCGTCAGGAGAAAGAATACGCCGAGTTGAGCCAGCAGGCCCAGAGACAACGAATCAGGCAAATGGTTGATGAGGC  600
 ProGluPheArgArgGlnLeuArgArgSerAlaArgArgGlnAlaArgGlnGluLysGluTyrAlaGluLeuSerGlnGlnAlaGlnArgGlnArgIleArgGlnMetValAspGluAla
                          His

     A                            A                                    A A      A                 a   a
CAAGGAGGAGGGCTTCCCTACCACTTCGGACGAGAAGGAGGCCTACTTCCTCGAGCAGGTCCAGGCTGGTGAGATCTTGGGCCAGGATCgtaagtgttttgccaaccaagcgagaagaag  720
 LysGluGluGlyPheProThrThrSerAspGluLysGluAlaTyrPheLeuGluGlnValGlnAlaGlyGluIleLeuGlyGlnAspPro
                                                      SerLys

                    a                                                 A       A         A
gaacggaacttccgggacatgtgtcggctactaacactccattcgtaacagCCACCAAGGCCATCGATGCCTCCCTCGCTTTCTACAAGGCCCTCAAGGTCTACCCTACGCCCGGCGACC  840
                                                    ThrLysAlaIleAspAlaSerLeuAlaPheTyrLysAlaLeuLysValTyrProThrProGlyAspLeu
                                                                      Thr     Thr

            A               A                                a          a                                    A   A A
TTATCAGCATCTACGACAAGACCGTTGCCAAGgtaagaggaatacgccgcatagcacgggtggaatgtttactgatccgacaaacactagCCCATCCTCGACATCCTCGCCGAGATGATC  960
  IleSerIleTyrAspLysThrValAlaLys                                                                ProIleLeuAspIleLeuAlaGluMetIle
             Asn                                                                                          Thr   Ile

            A                                 A       A                    A             A A    A T
GCTTACGACCCCAGCCTCAAAATTGGCACCAACTACACCGGCGGCGTCGATGTTGCCGAGCTCATGCGCGAGATGGCCTCCGCCCCCGGTGTTGGCCTCGACTAAACGAGTCCACCAAAT 1080
AlaTyrAspProSerLeuLysIleGlyThrAsnTyrThrGlyGlyValAspValAlaGluLeuMetArgGluMetAlaSerAlaProGlyValGlyLeuAsp
           Asn                               Ser      Ile                  Ile             IleSer   Asn

                              A       A                                T             A
ACAGCAAGAAGCATAACGCTAGGGAGAGTGACAACAATAGCCCACCTCGCAAGAAACGTCCGGGGGTATCAGTTGACGTTGCGGTGGTTCCCCGCAGTCCCTCAAAGGGGTTAGGGGAAT 1200

                             T  A T       A        A                                  A                    A
TGGGTGGTTACGGTTGTTGGGAAAACGGCGCGTGACGCACCAACGATGGCTTTTGTGGACGAAAGGAAGAAAGCTAGGAATAGTGGAAACAGGACGAGCATTCCAACAGACGACCCCGGA 1320
TCGATTTATACCCAGCCAGCTAGAAGAAAAAGGGGAGGCTAGCGGTCCTTTGCTTGTCTGACACATAACTCCCCTTTCAACGTACGTCCTTTGCACCGCACTTTGGCTCTCTCTCCCCGG 1440

TCAGTATTGCTCTCTCACGTCACCACTCTTGAACTCCCAACAGGTCAGCAGGTTTACGAGCACCACACTTCCTCCCTTATCATTGGCTGGCTGGTATCTCAACCGAGCGTTTGAGGGTCG 1560
AACGCTCACCCGAACTATCTACCTACCACCTTGTACAATATTTCTGTTCTGTTCTGTTCATGTTCCGGAGATGCGCGGGATGGTGGTGGTCTCCTATTTTATCCTGTAGCATCATGTACA 1680
TAGCGAAAATTGGGTCCTATCAGGATAACGGACGGTGATTTCTGTATGCGCGAACGGGATAGCAGGCCACTTCAGGGGTGCTTAATGAGACAAGAACGTAAGAAACGTATTGCCAATCTT 1800
CCCACTACGATC 1812
```

FIGURE 5.—Sequence of the resident RIPed allele in strain 28.17. Asterisks indicate the extent of the duplication (*i.e.*, the *mom-19* region cloned in pKSH6), which should serve as a target for RIP. The *Eco*RV and *Sau*3AI sites that define the extent of the cloned *mom-19* region in pKSH6 (see Figure 1) are underlined. The two small arrows below the sequence delineate the region that has been sequenced. Lower case letters indicate intron sequences. The complete sequence of the wild-type gene is shown. Bases above the wild-type sequence indicate the positions where mutations were found in the RIPed version of the resident gene found on the original host LG IV in strain 28.17. The amino acid sequence of the protein encoded by the wild-type gene is indicated immediately below the sequence. Amino acid alterations resulting from RIP mutations are shown under the normal protein sequence. Our sequence of the wild-type allele, as shown in this figure, revealed minor errors in the sequence previously entered in Genbank (accession number M80528). These changes have been reported to Genbank and are entered under the previous accession number as is the sequence of the RIP allele shown in this figure.

the MOM19 protein following growth as either heterokaryons without fpa or in the presence of uridine and 600 μM fpa. The latter conditions require that the nucleus containing the RIPed *mom-19* alleles be in a large majority in the cultures. Under such conditions the growth rate of both strains is reduced relative to controls as shown in Table 2. The inhibition of growth rate is also observed in liquid cultures (not shown). Mitochondria were isolated from these cultures, solubilized and subjected to polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membrane and probed with antibodies raised against the MOM19 protein. As shown in Figure 6, the MOM19 protein was undetectable in strain 28.17 when grown in the presence of fpa and uridine. When up to 200 μg of mitochondrial protein was

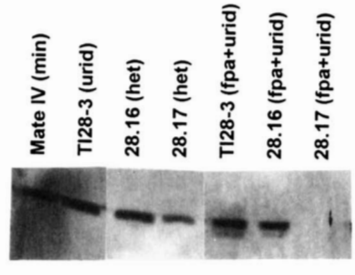

FIGURE 6.—Western blot analysis of RIP strains. Forty μg of mitochondrial protein were loaded per lane. Cells were grown in the indicated medium: min, minimal medium; urid, basal medium plus uridine; het, basal medium; fpa+urid, basal medium containing 600-μm fpa plus uridine. Following electrophoresis mitochondrial proteins were electroblotted to nitrocellulose membrane and decorated with polyclonal antiserum to MOM19.

loaded on the gels, we were still unable to detect MOM19 in mitochondria from these cultures (not shown). Although we have not rigorously quantified the levels of MOM19 in the different strains, inspection of the blots suggests that the amount of the protein is also reduced in 28.17 cultures grown as heterokaryons without fpa (Figure 6). The simplest explanation for this observation is that the heterokaryotic cells contain both RIPed and wild-type alleles of *mom-19* resulting in an overall reduction in MOM19 content. No changes in the amount of MOM19 protein were obvious in strain 28.16.

Rescue of *mom-19*^{RIP} mutant: We desired to obtain evidence that nuclei containing the RIPed *mom-19* allele could be rescued by transformation with a wild-type copy of *mom-19* to verify that the effects we observed were specific to *mom-19*. Both genomic and cDNA versions of *mom-19* were used to transform mutant 28.17. Successful transformation was qualitatively assessed by the appearance of rapidly growing colonies on plates containing basal medium plus uridine and 600 μM fpa. By this criterion, both genomic and cDNA clones of *mom-19* were capable of restoring rapid growth rate when transformed into spheroplasts of strain 28.17. In addition, three primary transformants were purified through one round of single colony isolation on basal medium containing uridine plus 600 μM fpa and examined for the presence of MOM19 protein in their mitochondria. Strain 17-43.1 was derived from transformation of strain 28.17 with a cosmid containing *mom-19* and an additional 35 kb of *N. crassa* genomic sequence. Strain 17-67.1 was derived by transformation with a plasmid containing *mom-19* and an additional 4 kb of genomic sequence. Strain 17-94.2 was obtained by transformation with a plasmid containing *mom-19* cDNA. All three strains were found to be incapable of growth on basal medium or basal medium containing tryptophan. However, they were able to grow on basal medium containing uridine or uridine and fpa. Thus, these strains are homokaryons containing the Host IV version of LG IV and do not contain the sheltering copy of *mom-19* from the Mate LG IV. As shown in Figure 7A, the mitochondria of all three transformed strains contain the MOM19 protein. The growth rate of these transformed strains was measured in "race tubes" (Davis and De Serres 1970) and compared with control strains. There was little difference between the transformants and control strains when grown on medium that does or does not contain fpa (Figure 7, panels B and C, respectively). The ability of cDNA clones to rescue the mutant phenotype is significant since it proves that extension of RIP from duplicated *mom-19* sequence into neighboring genes (Foss *et al.* 1991) is not responsible for the phenotypes observed.

Homokaryons containing the *mom-19*^{RIP} allele: The finding that the MOM19 protein was absent from

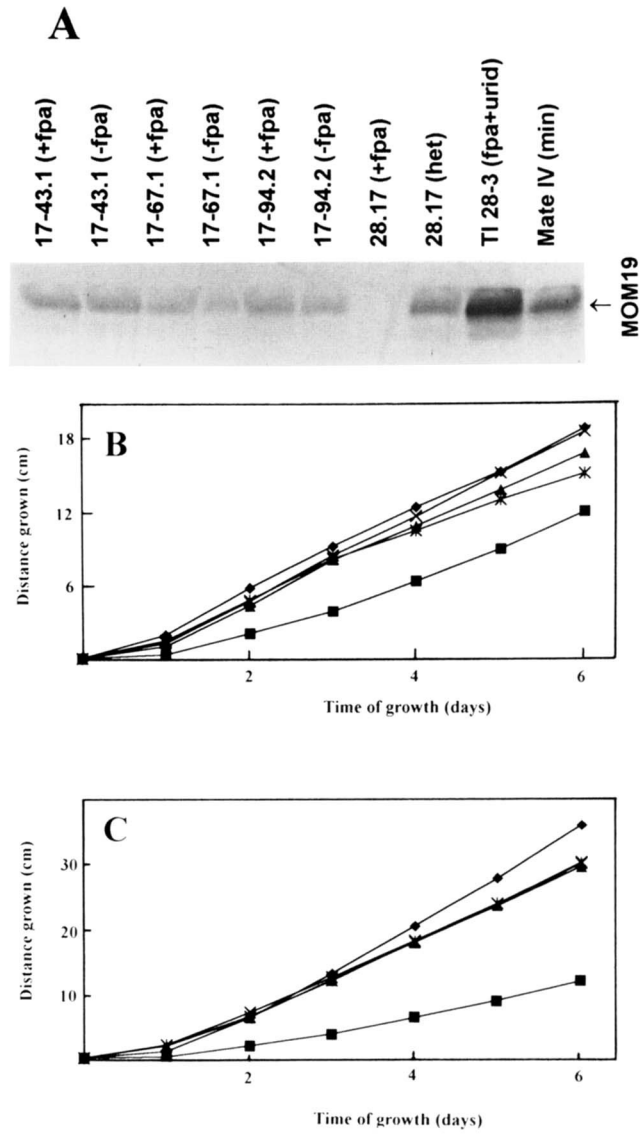

FIGURE 7.—Analysis of transformants. (A) Western blot of mitochondria isolated from transformants grown on basal medium plus uridine. The presence or absence of 600 μM fpa in the growth medium is indicated on the figure. The control strains were Mate IV grown in minimal medium, TI28-3 grown in basal medium plus uridine and fpa, 28.17 grown in basal medium as a heterokaryon (het), and 28.17 grown in basal medium plus uridine and fpa. Eighty μg of mitochondrial protein from each of the indicated strains were electrophoresed, transferred to nitrocellulose membrane and decorated with polyclonal antiserum to MOM19. (B) Growth of transformants 17-43.1 (▲), 17-67.1 (x) and 17-94.2 (★) and control strains TI28-3 (♦) and 28.17 (■) in race tubes containing basal medium plus uridine and 400 μM fpa. (C) Growth of transformants on medium lacking fpa. Symbols for the transformant growth curves 17-43.1, 17-67.1 and 17-94.2 are as in panel B. The transformants were grown on basal medium containing uridine. For a control to indicate the growth rate of the untransformed strain as a heterokaryon, strain 28.17 was grown on basal medium without fpa (♦). To indicate the slow growth rate when the RIPed nucleus is forced to predominate, strain 28-17 was grown on basal medium plus uridine and 400 μM fpa (■).

28.17 cultures grown in the presence of uridine and fpa suggested that the protein might not be essential for growth. To test this notion further, we reasoned

that if *mom-19* is an essential gene and strain 28.17 contains a null allele of the gene in the *pyr-1, trp-4*$^+$, *mtrR* nucleus of the heterokaryon, then it should not be possible to isolate homokaryons of that nucleus. To determine if such homokaryons could be isolated, we plated conidia from strain 28.17 on basal medium containing uridine but lacking tryptophan and picked 78 single colony isolates to tubes containing the same medium. These were allowed to conidiate and then tested for their ability to grow on basal medium. Isolates able to grow on basal medium must be heterokaryons, but isolates that cannot grow on this medium should be *pyr-1 trp-4*$^+$ homokaryons. We found that 64 of the colonies picked from the original plates were able to grow on basal medium, but 14 were unable to grow without added uridine. These 14 were presumably homokaryons containing only the RIPed version of *mom-19*. This interpretation was supported by showing that each of the 14 putative homokaryons was resistant to fpa. The *mom-19*^{RIP} homokaryons isolated in this fashion have a very slow growth rate and produce very few conidia. The density of mycelium formed in slants also appears to be thinner than with control strains. When we examined the growth of 12 of the homokaryons in race tubes, it was found that the strains exhibited a complex behavior. In addition to their slow rate of growth, all the isolates stopped growing after various distances down the race tube while controls continued to grow until the end of the tube was reached. However, many of the homokaryons began growth again, usually from a small sector of the old growth front, after several days without growth. For at least some isolates, the stop-start growth pattern was repeated through several cycles. The growth patterns of representative strains are shown in Figure 8A. The length of the period of growth in race tubes before the first cessation of growth is not a consistent characteristic associated with each of the individual homokaryotic strains since the amount of growth that precedes the stopped phase is progressively reduced when race tubes are inoculated with material from successive subcultures. This is shown in Figure 8B for strain M17-76, one of two homokaryons that grew over 35 cm when inoculated from the primary subculture of the strain. Hence, the organism is apparently capable of short-term growth without a functional *mom-19* gene but not sustained long-term growth. Unfortunately, we have been unable to generate sufficient material from these homokaryotic strains for further analysis.

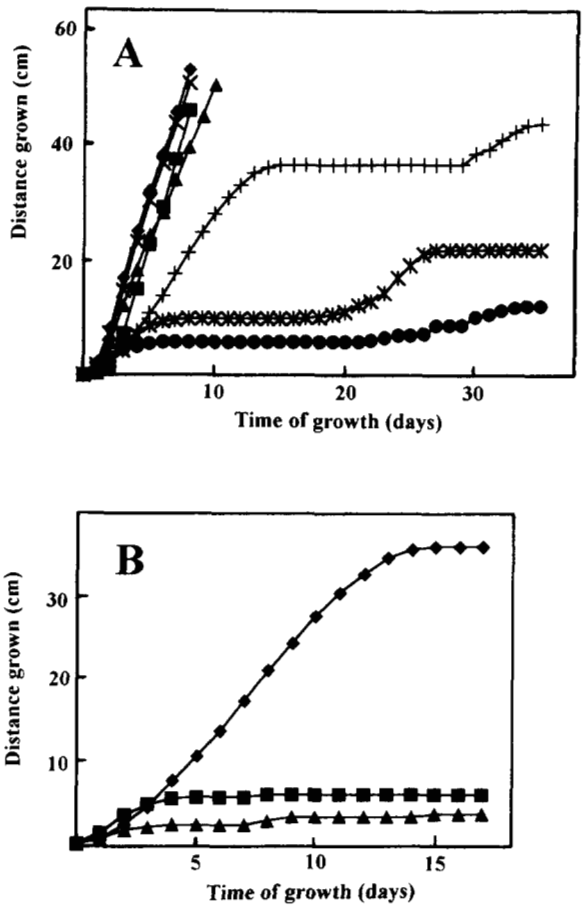

FIGURE 8.—Growth patterns of *mom-19*^{RIP} homokaryons in race tubes. (A) Growth was measured for 50 cm or for 35 days. Control strains were Mate IV grown on minimal medium (■), Host IV grown on basal medium plus uridine (♦), TI28-3 grown on basal medium plus uridine (▲) and 28-17 grown as a heterokaryon on basal medium (x). Three strains that represent the range of growth capacity and the stop-start growth behavior observed in *mom-19*^{RIP} homokaryons are also shown: M17-65 (★), M17-69 (●), M17-76 (+). These homokaryons were grown on basal medium containing uridine. (B) Effect of subculturing on extent of growth in race tube shown for homokaryon M17-76. Cultures were grown on basal medium plus uridine. First subculture (♦), second subculture (■), third subculture (▲).

DISCUSSION

We have used the technique of sheltered RIP to generate mutants of *mom-19*. In at least two of the heterokaryotic strains (28.16 and 28.17) isolated following the RIP cross, two different alleles of *mom-19*, one wild-type, the other containing many transition mutations characteristic of RIP, were demonstrated by sequence analysis of PCR products. This suggests that the heterokaryotic strains have exactly the genetic makeup predicted. To obtain the desired mutants, we examined LG IV complementing strains for their ability to grow on basal medium supplemented with uridine and fpa and for evidence of RIP as seen on Southern blots following digestion of genomic DNAs with appropriate restriction enzymes. Evaluation of these two criteria alone were sufficient to lead us to the correct strains. Thus, the method of sheltered RIP should be useful as a general technique for the isolation of mutants in genes that are essential or have a severe effect on the growth of the organism. Appropriate strains for each of the seven *N. crassa* linkage groups are available (METZENBERG and GROTELUESCHEN 1992a). Perhaps the major problem with

the method will be to obtain confirmation of RIP in those cases where target sequences are only mildly affected by RIP. Such a problem might occur when the target duplication is relatively small (SELKER 1990). Mild RIP could result in effective inactivation of a target gene, possibly by altering only one or two codons, but would be difficult to detect by examination for restriction site changes. In such cases the only way to demonstrate RIP may be by sequence analysis following PCR of target genes. In these instances it would be useful to isolate DNA from cultures grown under conditions selective for the RIPed nucleus since this will increase the ratio of RIPed to non-RIPed alleles.

We assume that the generation of 18 amino acid substitutions in strain 28.17 has effectively disrupted the gene and results in a nonfunctional gene product. This is supported by the observation that the polyclonal antibody used to detect the protein on Western blots reveals no protein in the mitochondria of 28.17 cultures grown in the presence of uridine and fpa. It seems likely that the altered protein is rapidly degraded, inefficiently sorted to mitochondria, or both. Although we cannot entirely eliminate the possibility that the protein retains a low level of activity despite lacking all antigenic sites found on the wild-type protein, this seems unlikely. The possibility that the phenotypic affects we observe in strain 28.17 are due to alterations in other genes, unintentionally altered during the RIP cross, is effectively eliminated by the observation that MOM19 and growth rates can be restored in cDNA transformants.

The inability to detect MOM19 in mitochondria from strain 28.17 grown in the presence of 600 μM fpa plus uridine suggests that MOM19 is not an absolute requirement for growth since the cells do grow, albeit at reduced growth rates, at this concentration of fpa in both slants (Table 2) and liquid cultures (not shown). The isolation of *mom-19*^{RIP} homokaryons supports the notion that MOM19 is not essential. However, the slow and rather complex growth behavior of the homokaryons makes it apparent that loss of MOM19 is insalubrious for the organism. One possible explanation for the range of growth behaviors in the different homokaryons is that mitochondria containing functional MOM19 are initially present in the culture and are diluted out as growth occurs. Such mitochondria would be present in the conidia formed from the original heterokaryotic culture used for plating. The length of time that each homokaryon continues to grow in the race tubes may be related to random factors that influence the number of mitochondria in different conidia and/or the amount of MOM19 present in the mitochondria of each initial isolate. It should also be noted that cessation of growth probably does not correspond immediately to the loss of MOM19, but rather to the depletion of essential components within the mitochondrion that are not replenished due to lack of MOM19. This might result in at least some delay in the appearance of phenotypic effects. It may also be that inefficient import via alternate routes, such as MOM72 or MOM22 can sustain slow growth for short periods. A possible explanation for the "stop-start" growth pattern of the homokaryons is that import via alternate routes may allow accumulation of essential proteins in mitochondria to occur very slowly. When a threshold level of these proteins is reached in the mitochondria of a particular cell, growth begins again. One possibility for what these proteins might be is nuclear encoded proteins involved in the maintenance or replication of mtDNA, since a similar pattern of stop-start growth has been observed previously in the so-called "stopper" extranuclear mutants of *N. crassa* (MCDOUGALL and PITTENGER 1966; BERTRAND and PITTENGER 1969, 1972). In these cases, the stop-start growth pattern has been explained by changing ratios of different forms of mtDNA in the culture (BERTRAND *et al.* 1980; GROSS, HSIEH and LEVINE 1984).

Mom-19 mutant strains should prove useful in understanding the functional role of the protein in the import process. In fact, in a detailed biochemical investigation (T. A. A. HARKNESS *et al.*, unpublished data) we have found that mitochondria isolated from MOM19 deficient cells exhibit a grossly altered morphology in that they lack cristae. The apparent lack of mitochondrial inner membranes is also reflected in a marked reduction of cytochromes and other inner membrane components like the ADP/ATP carrier, while the steady-state levels of most outer membrane and matrix components are virtually unchanged. On the other hand, protein import into isolated MOM19-deficient mitochondria is drastically reduced for most mitochondrial preproteins, regardless of the mitochondrial subcompartment to which they are normally sorted. Thus, the study of the properties of this mutant has already complemented and extended the understanding of the import process previously obtained by biochemical methods. The *mom-19* mutant should also be useful for investigating the assembly of the import apparatus. Furthermore, using sheltered RIP, we are now attempting to create mutants in other genes that encode proteins of the mitochondrial import machinery.

This work was supported by a grant from the Natural Sciences and Engineering Council of Canada to F.E.N., by grant GM08995 from the U.S. Public Health Service to R.L.M., and by grants from the Sonderforschungsbereich 184 and the Fonds der Chemischen Industrie to W.N. and R.L. T.A.A.H. was the recipient of a scholarship from the Alberta Heritage Foundation for Medical Research. F.E.N. is grateful for the support of the Alexander von Humboldt Foundation while on sabbatical leave in W.N.'s laboratory.

LITERATURE CITED

AKINS, R. A., and A. M. LAMBOWITZ, 1985 General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. **5:** 2272–2278.

ASCH, D. K., and J. A. KINSEY, 1990 Relationship of vector insert size to homologous integration during transformation of *Neurospora crassa* with the cloned *am* (GDH) gene. Mol. Gen. Genet. **221:** 37–43.

AUSUBEL, F. A., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, J. A. SMITH and K. STRUHL, 1992 *Current Protocols in Molecular Biology.* Greene and Wiley Interscience, New York.

BAKER, K. P., A. SCHANIEL, D. VESTWEBER and G. SCHATZ, 1990 A yeast mitochondrial outer membrane protein essential for protein import and cell viability. Nature **348:** 605–609.

BERTRAND, H., and T. H. PITTENGER, 1969 Cytoplasmic mutants selected from continuously growing cultures of *Neurospora crassa*. Genetics **61:** 643–659

BERTRAND, H., and T. H. PITTENGER, 1972 Isolation and classification of extranuclear mutants of *Neurospora crassa*. Genetics **71:** 521–533.

BERTRAND, H., R. A. COLLINS, L. L. STOHL, R. R. GOEWERT and A. M. LAMBOWITZ, 1980 Deletion mutants of *Neurospora crassa* mitochondrial DNA and their relation to the "stop-start" growth phenotype. Proc. Natl. Acad. Sci. USA **77:** 6032–6036.

BRADFORD, M. M., 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

CAMBAREri, E. B., B. C. JENSEN, E. SCHABTACH and E. U. SELKER, 1989 Repeat-induced G-C to A-T mutations in Neurospora. Science **244:** 1571–1575.

CULLEN, D., S. A. LEONG, L. J. WILSON and D. J. HENNER, 1987 Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. Gene **57:** 21–26.

DAVIS, R. H., and F. J. DE SERRES, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17:** 79–143.

FINCHAM, J. R. S., I. F. CONNERTON, E. NOTARIANNI and K. HARRINGTON, 1989 Premeiotic disruption of duplicated and triplicated copies of the *Neurospora crassa am* (glutamate dehydrogenase) gene. Curr. Genet. **15:** 327–334.

FOSS, E. J., P. W. GARRETT, J. A. KINSEY and E. U. SELKER, 1991 Specificity of repeat-induced point mutation (RIP) in Neurospora: sensitivity of non-Neurospora sequences, a natural diverged tandem duplication, and unique DNA adjacent to a duplicated region. Genetics **127:** 711–717.

FREDERICK, G. D., D. K. ASCH and J. A. KINSEY, 1989 Use of transformation to make targeted sequence alterations at the *am* (GDH) locus of Neurospora. Mol. Gen. Genet. **217:** 294–300.

GLASS, N. L., and L. LEE, 1992 Isolation of *Neurospora crassa A* mating type mutants by repeat induced point (RIP) mutation. Genetics **132:** 125–133.

GOOD, A. G., and W. L. CROSBY, 1989 Anaerobic induction of alanine aminotransferase in barley root tissue. Plant Physiol. **90:** 1305–1309.

GRIFFITHS, A. J. F., 1982 Null mutants of the *A* and *a* mating type alleles of *Neurospora crassa*. Can. J. Genet. Cytol. **24:** 167–176.

GRIFFITHS, A. J. F., and A. M. DELANGE, 1978 Mutations of the *a* mating-type gene in *Neurospora crassa*. Genetics **88:** 239–254.

GROSS, S. R., T. HSIEH and P. H. LEVINE, 1984 Intramolecular recombination as a source of mitochondrial chromosome heteromorphism in Neurospora. Cell **38:** 233–239.

KIEBLER, M., R. PFALLER, T. SÖLLNER, G. GRIFFITHS, H. HORSTMANN, *et al.*, 1990 Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. Nature **348:** 610–616.

KIEBLER, M., P. KIEL, H. SCHNEIDER, and W. NEUPERT, 1993 The mitochondrial receptor complex: a central role of MOM22 in mediating preprotein transfer from receptors to the general insertion pore. Cell **74:** 483–492.

LAEMMLI, U. K., 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

MCDOUGALL, K. J., and T. H. PITTENGER, 1966 A cytoplasmic variant of *Neurospora crassa*. Genetics **54:** 551–565.

METZENBERG, R. L., and J. S. GROTELUESCHEN, 1992a Disruption of essential genes in Neurospora by RIP. Fungal Genet. Newsl. **39:** 37–49.

METZENBERG, R. L., and J. S. GROTELUESCHEN, 1992b Restriction polymorphism maps of *Neurospora crassa*: update. Fungal Genet. Newsl. **39:** 50–58.

METZENBERG, R. L., J. N. STEVENS, E. U. SELKER and E. MORZYCKA-WROBLEWSKA, 1984 A method for finding the genetic map position of cloned DNA fragments. Neurospora Newsl. **31:** 35–39.

METZENBERG, R. L., J. N. STEVENS, E. U. SELKER and E. MORZYCKA-WROBLEWSKA, 1985 Identification and chromosomal distribution of 5S rRNA genes in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **82:** 2067–2071.

NEHLS, U., T. FRIEDRICH, A. SCHMIEDE, T. OHNISHI and H. WEISS, 1992 Characterization of assembly intermediates of NADH: ubiquinone oxidoreductase (complex I) accumulated in Neurospora mitochondria by gene disruption. J. Mol. Biol. **227:** 1032–1042.

PERKINS, D. D., 1984 Advantages of using the inactive-mating-type a^{m1} strain as a helper component in heterokaryons. Neurospora Newsl. **31:** 41–42.

PERKINS, D. D., 1992 *Neurospora crassa* genetic maps. Fungal Genet. Newsl. **39** (Suppl.): 153–162.

PFALLER, R., H. F. STEGER, J. RASSOW, N. PFANNER and W. NEUPERT, 1988 Import pathways of precursor proteins into mitochondria: multiple receptor sites are followed by a common membrane insertion site. J. Cell Biol. **107:** 2483–2490.

ROTHSTEIN, R., 1991 Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. **194:** 281–301.

SCHECHTMAN, M., 1986 A moderate-scale DNA prep for Neurospora. Fungal Genet. Newl. **33:** 45–46.

SCHNEIDER, J. C., and L. GUARENTE, 1991 Vectors for expression of cloned genes in yeast: Regulation, overproduction and underproduction. Methods Enzymol. **194:** 373–388.

SCHNEIDER, H., T. SÖLLNER, K. DIETMEIER, C. ECKERSKORN, F. LOTTSPEICH, *et al.*, 1991 Targeting of the master receptor MOM19 to mitochondria. Science **254:** 1659–1662.

SCHWEIZER, M., M. E. CASE, C. C. DYKSTRA, N. H. GILES and S. R. KUSHNER, 1981 Identification and characterization of recombinant plasmids carrying the complete *qa* gene cluster from *Neurospora crassa* including the *qa-1*$^+$ regulatory gene. Proc. Natl. Acad. Sci. USA **78:** 5086–5090.

SELKER, E. U., 1990 Premeiotic instability of repeated sequences in *Neurospora crassa*. Annu. Rev. Genet. **24:** 579–613.

SMITH, D. A., 1974 Unstable diploids of Neurospora and a model for their somatic behavior. Genetics **76:** 1–17.

SMITH, D. A., 1975 A mutant affecting meiosis in Neurospora. Genetics **80:** 125–133.

SÖLLNER, T., G. GRIFFITHS, R. PFALLER, N. PFANNER and W. NEUPERT, 1989 MOM19, an import receptor for mitochondrial precursor proteins. Cell **59:** 1061–1070.

SÖLLNER, T., R. PFALLER, G. GRIFFITHS, N. PFANNER and W. NEUPERT, 1990 A mitochondrial import receptor for the ADP/ATP carrier. Cell **62:** 107–115.

STABEN, C., B. JENSEN, M. SINGER, J. POLLOCK, M. SCHECHTMAN, *et al.*, 1989 Use of bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora crassa* transformation. Fungal Genet. Newsl. **36:** 79–81.

Communicating editor: R. H. DAVIS