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CLONING AND CHARACTERIZATION OF GENES INVOLVED IN THE
REGULATION OF THE ALTERNATIVE OXIDASE AND MITHOCHONDRIAL
DNA REPLICATION IN NEUROSPORA CRASSA

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CLONING AND CHARACTERIZATION OF GENES INVOLVED IN THE REGULATION OF THE ALTERNATIVE OXIDASE AND MITHOCHONDRIAL DNA REPLICATION IN *NEUROSPORA CRASSA*

By

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ABSTRACT

CLONING AND CHARACTERIZATION OF GENES INVOLVED IN THE REGULATION OF THE ALTERNATIVE OXIDASE AND MITOCHONDRIAL DNA REPLICATION IN *NEUROSPORA CRASSA*

By

Tak Ko

The mitochondria of *Neurospora crassa* contain two respiratory pathways, the normal electron-transport chain and an inducible cyanide-resistant alternative oxidase pathway. In *Neurospora*, the assembly of the alternative oxidase pathway requires the expression of two genes, *aod-1*, which encodes the alternative oxidase, and *aod-2*, which is involved in the transcriptional regulation of *aod-1*. The objective of this work was the cloning and characterization of the *aod-2* gene. Crosses between strains that carried markers in linkage group IIR (*thr-3* and *arg-5*) suggest that *aod-2* is located 14 map units from *thr-3* and 3 map units from *arg-5*, and to the right of a translocation, TALS176. An attempt was made to isolate the *aod-2*$^+$ DNA in two different ways: chromosome walking, and by complementation of an *aod-2* mutant using *aod-1* promoter-driven expression of the bacterial hygromycin resistance gene as a method for selecting transformants. An expression vector that contains the putative promoter upstream of a bacterial hygromycin-resistance gene as a reporter has been constructed. However, a working selection system that can be used to select *aod-2*$^+$ by complementation of an *aod-2* mutant is not available yet. Similarly, a contig of cosmids was constructed for the region of

linkage group IIR covering the vicinity of the *aod-2* locus. Transformation of the *aod-2* mutant with cosmids from this contig, however, so far has failed to detect the gene.

Most of the proteins involved in mitochondrial gene replication and expression are encoded by nuclear genes. Included in this group of proteins is DNA polymerase-γ, which is part of the complex involved in the replication of mitochondrial DNA. I have cloned, sequenced and partially characterized the gene (*mip-1*) that codes for the presumptive mitochondrial DNA polymerase of *Neurosopra crassa*. Sequence analysis showed that *mip-1* includes a 3918-nucleotide open reading frame encoding a 1305 amino-acid, 146 kDa protein that has a mitochondrial targeting element at the amino terminus. The gene is transcribed to a 4.5-kb mRNA. RFLP mapping located the gene in linkage group III between *pro-1* and *ad-2*. A comparison of the nine available DNA polymerase-γ sequences revealed several highly conserved sequence blocks, and that the polymerase domain is more highly conserved than the exonuclease domain. The *N. crassa* and *S. cerevisiae* polymerase-γ polypeptides have long C-terminal extensions that are not found in any of the available homologous proteins from other species.

Dedicated in the fond of memory of my father

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CHAPTER 1.

Approach to clone the *aod-2* alternative oxidase regulatory gene of *Neurospora crassa*

1.1 INTRODUCTION

1.1.1 Respiratory Pathways in Plants and Fungi

Mitochondria are the sites of oxidative metabolism in eukaryotes. They carry out the energy capturing reactions during cellular respiration. While the mitochondria of animal cells contain a single, cytochrome-dependent respiratory system which is inhibited by cyanide and antimycin-A, higher plants, fungi, algae, and some protistes, are known to have two respiratory pathways. One of those two systems is the normal cytochrome pathway, which is cyanide and antimycin-A sensitive, and the second pathway is an oxidase that is insensitive to cyanide and antimycin-A, but is inhibited by hydroxamic acids (Henry and Nyns 1975). This pathway is commonly known as the "alternative pathway".

The alternative oxidase has been demonstrated to exist in some yeast, including *Hansenula anomala*, (Henry and Nyns, 1975; Sakajo *et al.*, 1991; 1993) and *Moniliella tomentosa* (Hanssens *et al.*, 1974), protists, particularly in *Trypanosoma* (Evans and Brown, 1973; Hill, 1976; 1978), green algae, such as *Chlamydomonas* (Sargent and Tylor, 1972), filamentous fungi, including *Neurospora*, *Cryphonectria* and *Aspergillus* among others, and in higher plants, including *Sauromatum guttatum*, *Arabidopsis*, soybean, potato, rice, petunia and tobacco (Rhoades and McIntosh, 1991; McIntosh, 1994; Vanlerberghe *et al.*, 1995). However, there are no reports of the alternative-oxidase activity in animal mitochondria.

The cytochrome chain of *N. crassa* is similar to that of higher eukaryotes (Figure 1-1) in that it contains enzymes and multiprotein complexes that perform key functions in oxidative phosphorylation. These multiprotein complexes are responsible for electron transfer between the various TCA cycle substrates and molecular oxygen. The four major complexes are the NADH:ubiquinone reductase (complex I), succinate:ubiquinone reductase (complex II), ubiquinol:cytochrome *c* reductase (complex III), and cytochrome *c* oxidase (complex IV). Complex I, III and IV are sites of proton pumping across the inner mitochondrial membrane. The proton gradient is subsequently dissipated by the F_0F_1-ATPase (complex V) and this process is coupled with ATP production (Hatefi, 1985). When electrons flow through the cytochrome pathway, energy is conserved in the form of an electrochemical gradient across the inner mitochondrial membrane which is impermeable to protons (Elthon and Stewart, 1983). The alternative pathway diverges from the cytochrome pathway at the ubiquinone pool and is not linked to oxidative phosphorylation (Palmer, 1976).

Electron flow through the alternative pathway involves the shunting of electrons from the main pathway at the level of the ubiquinone pool (Moore and Siedow, 1991). The alternative-oxidase pathway consists solely of a ubiquinone oxidase that transfers electrons from reduced ubiquinone to oxygen in a reaction that produces water. The alternative-oxidase pathway bypasses two energy-conservation sites of the standard electron transport chain; thus electron flow through the alternative pathway is considered energetically wasteful. The alternative oxidase activity is tightly associated with the inner mitochondrial membrane and appears to represent an integral membrane protein or protein complex, but a protonmotive force does not develop during electron flow through this pathway (Moore *et al.*, 1978).

1.1.2 Structure of the Alternative Oxidase

The recent years, there has been a considerable advance in the understanding of the structural features of the alternative-oxidase. The elucidation of the structure of this respiratory pathway began with the generation of an antibody that inhibited cyanide-insensitive respiration in isolated mitochondria from *Sauromatum guttatum* (Elthon and McIntosh, 1987; Elthon *et al.*, 1989). Immunoblotting experiment showed three proteins having molecular mass of 35, 36.5, and 37 kDa, in *S. guttatum* (Elthon *et al.*, 1989). Experiments using an inducer, chloramphenicol, which inhibits mitochondrial proteins synthesis, revealed that the antibody recognized two polypeptides (37 and 36.5 kDa) in the mitochondria when the alternative oxidase is induced in the *N. crassa* (Lambowitz *et al.*, 1989). The specificity of the antibody was confirmed through the observation that the polypeptides were missing in some of the alternative oxidase deficient mutants of *Neurospora*. The *S. guttatum* antibodies also recognized one single polypeptide of 36 kDa in *Hansenula anomala* treated with Antimycin-A (Sakajo *et al.*, 1993). At present, neither the nature nor the physiological significance of the multiple bands that were observed in plants and *Neurospora* is understood clearly.

The antibody against the alternative oxidase protein was also used to isolate a cDNA clone that encoded the alternative oxidase protein in *Sauromatum guttatum*

(Rhoades and McIntosh, 1991). Subsequently, cDNA and/or genomic DNA sequences encoding the alternative oxidase were obtained from *Arabidopsis* (Kummer and Soll, 1992), soybean (Whenlan *et al.*, 1993), tobacco (Vanlerberghe and McIntosh, 1994), the yeast *Hansenula anomala* (Sakajo *et al.*, 1991), and *N. crassa* (Li *et al.*, 1996). The alternative oxidase polypeptides are approximately 350 amino acids long, and has an N-terminal extension that targets the protein to mitochondria. The alternative oxidase of *N. crassa* is a chain of 362 amino acids and the mitochondrial targeting sequence is cleaved by the mitochondrial import machinery in front of the leucine residue at protein 65, which the predicted to be the start of the mature protein (Li *et al.*, 1996).

1.1.3 Possible Functions of the Alternative Oxidase

Although the cyanide-resistant alternative-oxidase pathway was first described over 65 years ago (Genevois 1929), and much progress has been made towards a clear understanding of the mechanisms involved in the synthesis and activity of the complex, its physiological role remains obscure. There are at least five hypotheses that have been formulated to explain the physiological role of alternative oxidase pathway.

The first hypothesis is thermogenesis during flowering in *S. guttatum*, the voodoo lily (Meeuse, 1975; Raskin *et al.* 1987). This is the only confirmed role for the alternative-oxidase pathway. In this plant, the aroid spadix tissue contains mitochondria that have a high amount of a cyanide-resistant oxidase that results in a very high rate of respiration during anthesis. The energy that is released as heat during this respiration results in volatilization of compounds that attract pollinating insects. The thermogenic activity is induced by salicylic acid (Raskin *et al.* 1987).

The second hypothesis is that the alternative-oxidase pathway may provide an energy overflow mechanism which allows operation of the TCA cycle independent of ATP synthesis for the production of intermediates required in other metabolic processes (Lamber, 1982).

The third hypothesis postulates that alternative oxidase might generate heat to allow maintenance of respiratory function at low temperatures which would impair the operation of the cytochrome pathway (Laties, 1982). This suggestion was based on the observation that plant mitochondria increased the activity of alternative oxidase in response to low, non-freezing temperatures, and that electron flow through the alternative-oxidase pathway is less sensitive to reduced temperature than the main cytochrome pathway (Yoshida and Tagawa, 1979). This notion has been supported recently by the finding that the capacity of the alternative-oxidase pathway was increased in tobacco cells that were transferred from higher to lower temperatures (Vanlerberghe and McIntosh, 1992).

The fourth possibility is that the alternative oxidase defends organisms against respiratory inhibitors that are produced by competing organisms in the environment (Lambowitz and Zannoni, 1978). Many organisms have been shown to be able to produce cyanide and other compounds as secondary metabolites, which can act as respiratory inhibitors. These organisms might themselves be expected to have a respiratory pathway which is insensitive to these compounds (Lloyd and Edwards, 1977).

The fifth possible role of alternative oxidase is that it operates as an antioxidante defense mechanism (Purvis and Schewfelt, 1993; Popov *et al.* 1997). It was shown that inhibition of alternative oxidase with salicyl hydroxamte and propyl gallate stimulates H_2O_2 production by mitochondria oxidizing succinate. Thus, the alternative oxidase might be involved in limiting the level of reactive oxygen species produced in stressed and senescing plant tissues.

1.1.4 Regulation of the Activity and Synthesis of the Alternative Oxidase

In plants, there are two mechanisms that are involved in the regulation of alternative oxidase activity. One occurs at the protein level and depends on two factors, metabolites and the redox status of an intermolecular disulfide bond in the homodimer complex (Umbach and Siedow, 1993). In the presence of pyruvate, alternative oxidase activity is stimulated (Umbach *et al.*, 1994). Other metabolites, such as hydropyruvate, glyoxylate, α-keto acid, succinate, and malate have the same effect on the activity (Umbach *et al.*, 1994). In plant mitochondria, the alternative oxidase polypeptide appears to exist as a mixed population of covalently associated and noncovalently associated dimers. Dimers in which the two subunits are linked by a disulfide bond are less active than dimers in which this disulfide bond has been reduced. However, disulfide bridging in dimers does not occur in the fungal alternative oxidases (Li *et al.* 1996).

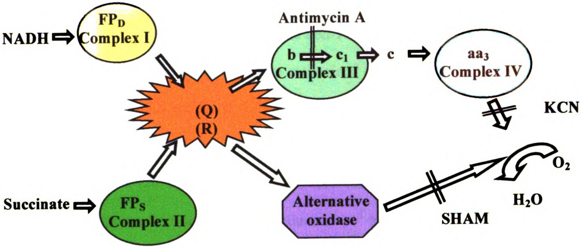
The second type of regulation varies the amount of alternative oxidase protein that is present in the membrane. This is the primary means of regulation of alternative oxidase activity in fungi. In these organisms, the alternative oxidase pathway is not active unless the cytochrome pathway is impaired (Slayman, 1977; Lambowitz and Zannoni, 1978). The biogenesis of the alternative oxidase pathway in *N. crassa* requires at least two genes, *aod-1*, which encodes the alternative oxidase subunit polypeptide, and *aod-2*, which is involved in the transcriptional regulation of *aod-1* (Bertrand *et al.* 1983; Li *et al*, 1996). In *N. crassa*, the existence of an alternative oxidase was first postulated through the observation of cyanide insensitive respiration in the [*poky*] cytoplasmic mutant, which is deficient in *a*- and *b*-type cytochromes (Tissiers *et al.* 1953; Lambowitz *et al.* 1972). The alternative oxidase was found to be inducible in wild-type strains of *N. crassa* by treatments that impair the cytochrome pathway (Lambowitz and Slayman, 1971), such as chloramphenicol, which inhibits mitochondrial protein synthesis (Lambowitz *et al.* 1972); oligomycin, an inhibitor of mitochondrial ATPase activity (Szakacs and Bertrand, 1976; Szakacs, 1978); antimycin A, an inhibitor of electron transport (Lambowitz and Slayman, 1971); and starving wild-type cultures for copper, which is an essential factor for the formation of cytochrome *c* oxidase (Schwab 1973). These observations suggest that the regulation of the alternative pathway must be achieved in *N. crassa* at the level of transcription and/or translation rather than by regulation of the enzymatic activity (Li *et al*, 1996). Experiments using an inhibitor of nuclear RNA synthesis, actinomycin-D, suggested that the induction of the alternative pathway is dependent on the transcription of nuclear genes (Edwards and Unger 1978).

The induction of alternative-oxidase activity in *N. crassa* requires at least two genes. From circumstantial evidence and nucleotide sequence data, it was deduced that *aod-1* encodes the alternative oxidase and *aod-2* is involved in the regulation of the expression of the *aod-1* gene (Bertrand *et al.* 1983). The *aod-1* gene is located at 23 map units to the left of *trp-4* gene in linkage group IV (Bertrand *et al.* 1983). This gene has been cloned recently (Li *et al.* 1996) and was shown to encode a polypeptide that is homologous to alternative oxidase proteins from other organisms. The *aod-2* gene is located on linkage group II (Bertrand *et al.* 1983). It was previously suggested that the function of *aod-2* was to encode either a component that regulates the synthesis of the alternative oxidase or a protein required for the stable accumulation of the *aod-1* polypeptide (Bertrand *et al.* 1983; Lambowitz *et al.* 1989). Subsequently, it was shown that the level of *aod-1* mRNA remains low in the *aod-2* mutant when it is grown under inducing conditions (Li *et al.* 1996). This result confirmed that the *aod-2* gene product is a factor involved in the transcriptional control of the *aod-1* gene.

The regulation of the alternative oxidase gene is of interest because it provides a model system for studying the mechanism of communication between mitochondria and the nucleus. Thus, cloning and characterization of the *aod-2* gene, which is known to affect the transcription of *aod-1* in response to the functional state of mitochondria, will provide information about this mechanism of mitochondrial/nuclear communication. *N. crassa* is an ideal organism for the study of alternative oxidase because wild type strains normally respire exclusively through the cyanide-sensitive, cytochrome-mediated pathway, but the alternative pathway can be induced by chemical or genetic inhibition of the cytochrome pathway (Lambowitz and Slayman, 1971). Furthermore, the genetic system of *N. crassa* has been characterized and several mutants deficient in alternative oxidase have been isolated (Szakacs and Bertrand, 1976) and in 1978 Edwards and Unger discovered that the transcription of nuclear gene is required for the induction of alternative oxidase activity.

The *aod-2* gene is not easily cloned because it is a regulatory gene and no equivalent gene has been identified or cloned from any other organism, thus eliminating the possibility of generating a PCR product by amplification of part of the gene with degenerate primers. Furthermore, mutations in this gene do not produce a deleterious phenotype, making it virtually impossible to clone it by gene replacement. Thus, an attempt was made to obtain the *aod-2* gene in two different ways: complementation of an *aod-2* mutant using *aod-1* promoter-driven expression of the bacterial hygromycin resistance gene (*hph*) and chromosome walking.

Figure 1-1. Electron transport pathway in *N. crassa*.



1.2 MATERIALS AND METHODS

1.2.1 *Neurospora* and *E. coli* strains and growth conditions

Wild type *N. crassa* 74-R23-1A and *aod-2* mutant strains (Table 1-1) were used in this study. All *N. crassa* strains were maintained on Vogel's medium (Vogel, 1956; Davis and Serres 1970) solidified with 1.5% w/v agar. Liquid cultures were grown in a reciprocating-shaker incubator. Vegetative cultures of all *N. crassa* strains were grown at room temperature (26°C) in the light to induce conidiation. Mycelia for respiration studies were grown from a conidial inoculum of 10^6 to 10^7 conida/ml in 25-ml shaker flasks containing 5 ml of Vogel's liquid medium. The flasks were shaken for 12 to 14 hr, and then 2-ml aliquots were removed from each flask for respiration studies.

E. coli DH5α strain was used as a host for all pBluscript-derived recombinant plasmids (Table 1-2). The bacterial strain was grown in L-broth (Maniatis *et al.*, 1982) at 37°C in a shaker incubator. Ampicillin was added to a final concentration of 50 μg/ml as required for selection of antibiotic resistance.

Table 1-1. *N. crassa* strains used in this study. Ant : antimycin sensitive

	Strain	Isolation no.	*Genotype*	
aod-1-1	7001	NSA-95	*aod-1, a*	
	7002	NSAK-33	*aod-1, pan-1, a*	
	7003	NSAK-44	*aod-1, pan-1, A*	
	7004	NSA-MA 6.40	*aod-1, nic-1, al-2, A*	
aod-1-2	7021	NSK-1	*aod-1, a*	
	7022	NSK-3	*aod-1, inos, al-3, a*	
	7023	NSKZ-39	*aod-1, pan-1, A*	
	7024	NSK-MA 18.2	*aod-1, nic-1, al-2, A*	
aod-1-3	7041	NSG-9	*aod-1, A*	clumpy conidiating
	7042	NSG-13	*aod-1, a*	clumpy conidiating
	7043	NSG-105	*aod-1, pan-1, A*	
	7044	NSGK	*aod-1, pan-1*	
aod-2-4	7061	NSBA-19	*aod-2, A*	slow conida
	7062	NSBA-24	*aod-2, a*	slow conida
	7063	NSBAN-9	*aod-2, nic-1, al-2, A*	
	7064	NSBAN-4	*aod-2, nic-1, al-2, a*	
	7065	NSBAN-12	*aod-2, nic-1, al-2, a*	
ant	7201	ant-3	*inos*	
	7202	ant-1-4	*aod-2, pan, A*	
	7203	ant-1		
	7204	ant-7	*aod-1, al-3*	
	7205	ant-7	*aod-1, al-3*	
	7206	ant-7	*aod-1, al-3*	
	7207	ant-7-7		frameshift
	7208	ant-6-122	*aod-1, trp-4*	
	7209	ant-6-122	*aod-1, trp-4*	
	7210	ant-1-28	*pan-2, trp-4*	
	7211	ant-1-11		
	7212	ant-1		
	7213	ant-1-27	*pan-2, trp-4*	
	7214	ant-1-27		
	7215	ant-7-40	*pan-2, al-3*	
	7216	ant-6-59	*aod-1, pan-2, trp-4*	aa substitution
aod+	4044		*met-1, A*	
	4045		*pdx—1, cyc-4, A*	
	4046		*pyr-1, arg-2, A*	
	4008-50		*a*	
	4115	27947 Y8743m Ltd37	*arg-5, pe, fl, trp-3, A*	
	4116	27947 Y8743m Ltd37	*arg-5, pe, fl, trp-3, a*	
	7194	27947 C167	*arg-5, aro-3, A*	
	7196	UM107 F29 P2420	*arg-12, ure-3, un-20, a*	
	2000	T28-M2 Y7655	*nuc-2, aro-1, A*	
	462		*his-3, A*	
	7626	RLM	*his-3 mtr, a*	

1.2.2 Preparation of competent *E. coli* cells for transformation

Bacterial transformation was done by standard procedure described by Maniatis *et al* (1982). Competent cells were preserved at –80°C in 1.5-ml micro tubes containing 0.5 ml of L-broth supplemented with 36% glycerin, 12%PEG (MW8000), 12 mM-$MgSO_4 7H_2O$ and sterilized by filtration.

1.2.3 Recombinant plasmids and vectors

The plasmid pBluscript KS+ (Strategene) was used as a cloning vector in this study. Table 1-2 provides the lists of the recombinant plasmids obtained from others or constructed in this study. The cosmid clones isolated during chromosome walks are shown in Table 1-2. Cosmid clones are named by their position in the microtiter plates of the library.

Table 1-2. Recombinant plasmids constructed and the cosmid clones isolated during chromosome walks in this study. The Orbach/Sachs cosmid library of *N. crassa* genomic DNA in pMOcosX vector, which has a dominant selectable markers for fungi (hygromycin resistance) and *E. coli* (ampicillin resistance), and pSV50 cosmid library of *N. crassa* genomic DNA (Volmer and Yanofsky 1986) in vector pSV50 carrying a selectable markers for fungi (benomyl resistance) were used for chromosome walking.

Cosmids or palsmids	Description
pAOGE-1	8 kb *Eco*RI fragment of the *aod-1* gene cloned in pBluscript
pDV8H+	Contains 2.5 kb *N. crassa his-3* gene
pCSN43	Contains hygromycin resistance gene (*hph*) and transcription termination sequence of *trpC* from *Aspergillus nidulans*
pHPH	pBluescriptKS with 2-kb segment of the *hph* gene and terminator sequence of *trpC*
pHH	pHPH with *N. crassa his-3* gene.
pHHm	pHH with mutated *Spe*I site.
pHHma	pHHm with 20 bp-long adaptor at *Cla*I site
pTAK28, pTAK29	Final construct contains sense orientation of 6-kb upstram region of *aod-1*, *hph* and *his-3* in pBluscriptKS.
pTAK54, pTAK61	Final construct contains antisense orientation of 6 kb upstram region of *aod-1*, and sense orientation of *hph* and *his-3* in pBluscriptKS.
G3:10C	pMOcosX cosmid library
G21:12E	pMOcosX cosmid library
X24:12B	pMOcosX cosmid library
G1:1C	pMOcosX cosmid library
G1:11H	pMOcosX cosmid library
25:1D	pSV50 cosmid library
X2:7C	pMOcosX cosmid library
X25:7A	pMOcosX cosmid library
X10:12G	pMOcosX cosmid library
G8:11H	pMOcosX cosmid library
G7:3D	pMOcosX cosmid library
X12:5D	pMOcosX cosmid library
G5:2D	pMOcosX cosmid library
YAC 2:6D	*N. crassa* YAC library

1.2.4 Rapid mini-scale plasmid/cosmid DNA isolation

Mini-scale plasmid/cosmid DNA preparations from 1.5 ml culture samples were done by alkaline lysis (Sambrook *et. al.*, 1989) or by using the Quantum Prep Plasmid Miniprep Kit (BIO-RAD) or the Wizard Mini-prep kit (Promega) as described by the manufacturers.

1.2.5 Storage and plating of *N. crassa* genomic DNA libraries

Two cosmid libraries and/or YAC library were used in this study. The two cosmid libraries were the Orbach/Sachs cosmid library of *N. crassa* genomic DNA in the pMOcosX vector, which has a dominant selectable markers for fungi (hygromycin resistance) and *E. coli* (ampicillin resistance) and the pSV50 cosmid library of *N. crassa* genomic DNA (Volmer and Yanofsky 1986) in vector pSV50 carries selectable markers for fungi (benomyl resistance) and *E. coli* (ampicillin resistance). Cosmid libraries were maintained at –80°C in separate 96-well microtiter plates containing LB medium supplemented with ampicillin.

For the wild-type *Neurospora crassa* genome, a yeast artificial chromosome (YAC) library (Centola and Carbon 1994) was obtained from the Fungal Genetics Stock Center (FGSC). The YAC library was stored in 24 microtiter plates in YPD medium (see appendix) at –80°C. The library contained 2204 clones with inserts of *N. crassa* genomic DNA averaging about 170 kb and representing about 8.7 genome equivalents (Centola and Carbon 1994).

1.2.6 Chromosomal walking through the *N. crassa* genomic DNA cosmid library

The Orbach/Sachs pMOcosX cosmid library of *N. crassa* genomic DNA was replica plated in 50 microtiter dishes. Each cosmid clone was grown in 200 μl selection medium (LB-amp) at 37°C for about 16 hr. DNAs were prepared from the 50 pools cosmid, each containing 96 clones in a microtiter plate. The pool DNAs were digested with *Eco*RI restriction endonuclease. The restriction fragments were separated by electrophoresis through 0.8% agarose gels and transferred to nylon membranes for Southern blot analysis to screen for homology to probes of relevant sequences. Plates containing positive clones were replicated onto new microtiter plates, and 12 column pools and 8 row pools of clones from each plate were used to prepare DNA for identification of the specific address of the desired clone by hybridization. The walk was extended with end-specific riboprobes transcribed from the T3 or T7 RNA polymerase promoters contained in the vector. RNA probes were labeled by using the Dig-RNA Labeling Kit (Boehringer Mannheim). Cosmids were isolated as described in section 1.2.4.

1.2.7 Measurement of O_2 consumption by *N. crassa* mycelium

Respiration by intact mycelia was measured using a Clark electrode with an YS1 Model 53 Biological Oxygen Monitor (Yellow Spring Instruments Co.) as described by

Lambowitz and Slayman (1971). KCN (0.1 M) was dissolved in water and SHAM (0.33 M) was dissolved in 95% ethanol. Stock solutions of these inhibitors were prepared freshly just before their use. 25 μl of each stock was added to 3 ml cultures in liquid Vogel's medium in the closed oxygen monitor vessel.

1.2.8 Induction of the alternative oxidase by inhibitors of oxidative phosphorylation

For the induction of the alternative oxidase, chloramphenicol (final concentration of 5 mg/ml, Sigma) and different final concentrations (0.05-1.0 μg/ml) of alcoholic solutions of oligomycin (Sigma) and antimycin A (Sigma) were added as alcoholic solution to 5 ml of 8-hr shaking liquid cultures and 4-hr further growth was allowed for the induction of the alternative oxidase. Respiration studies were done after a total of twelve hours of growth.

1.2.9 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels by the GlassMax™ DNA isolation Matrix system (GIBCO BRL) or by a modified gel electrophoresis method. In the modified gel electrophoresis system, purification was performed through the use of high melting point agarose. After the DNA was electrophoresis through 0.8-1.5% gels, the DNA was stained with ethidium bromide. Then some TBE buffer was removed from the gel box until it reached the top of the agarose-gel. A piece of 3MM paper and a dialysis membrane were inserted 5 mm away and on the cathode side of the desired fragment. These 3MM paper and dialysis membrane were cut 1 mm longer width of the DNA band on both sides. The 3MM paper was inserted into the gel close to the DNA fragment and the dialysis membrane was placed behind the 3MM paper. Subsequently, electrophoresis was performed for 30-60 minutes. The desired DNA fragments were eluted from the 3MM paper in 150 µl of TE solution.

1.2.10 PCR approach to cloning of *arg-5* from *N. crassa*

Genomic DNA was prepared from *N. crassa* according to the method of Lee and Taylor (1990). Figure 2 shows the amino-acid sequence comparison of the acetylornithine aminotransferases from *Alnus glutinosa* (Y08680), *Synechocystis sp.* (D90904), *E. coli* (M32796), *Saccharomyces cerevisiae* (M32795) that were used to design degenerate primers corresponding to the conserved EANEAA (5'-primer) and QGEGGV (3'-primer) amino-acid sequences. These primers were expected to produce a PCR product of about 280 bp if no intron is present in the portion of the *N. crassa arg-5* gene. PCR reactions were initially carried out using varying concentrations of $MgCl_2$ (0.5-5 µl of a 25 mM stock), and varying annealing temperatures. In general, the best results were obtained with the following reaction conditions: 100 ng of *N. crasssa* genomic DNA; 5 µl of 10X reaction buffer (Gibco); 2-3 µl of $MgCl_2$ (25 mM); 1 µl of dNTP mix (10 mM) and 1µl of each primer stock (20 µM); 0.5 µl of Taq polymerase (Gibco-5 U/µl); distilled water to give a total volume of 50 µl. Amplification was performed in a program consisting of 1 min at 94°C, annealing at 52°C for 1 min, and extension at 72°C for 1 min. This program was repeated through 35 cycles, followed by 5 min at 72°C to ensure completion of products. Amplified fragments were analyzed by electrophoresis in a 1.5 % agarose gel (Figure 1-16) and used for the cloning.

Bands of the appropriate sizes (over 1 kb, about 0.6 kb, and below 400 bp) were recovered from agarose gels as described in section 1.2.9, precipitated with ethanol and used for cloning. These gel purified PCR products were ligated into the plasmid vector pBluscript KS+ (Stratagene) using standard protocol (Sambrook *et. al.*, 1989) and sequenced using T3/T7 primers.

The Orbach/Sachs pMOcosX cosmid library of *N. crassa* genomic DNA was screened by Southern hybridization and by PCR with degenerate primers. Cosmids were isolated using the Quantum Prep Plasmid Miniprep Kit (BIO-RAD Inc.).

1.2.11 DNA Sequencing and Gene Analysis

DNA sequencing was performed through the use of a ABI Prism DNA sequencer. Analysis of DNA and protein sequences was performed by using the program of DNASTAR and the amino acid sequences of the acetylornithine aminotransferase from different species were aligned using the CLUSTAL program of DNASTAR Megalign. Some minor adjustments in the alignment were made by visual inspection.

1.2.12 Restriction Fragment Length Polymorphism (RFLP) mapping

The location of isolated cosmids on *N. crassa* was determined by RFLP mapping (Nelson et al. 1998). Genomic DNA was prepared from *N. crassa* strains #4450–4488 (Fungal Genetics Stock Center) and the Mauriceville and Oak Ridge parents according to the method of Lee and Taylor (1990) and 5 µg of each DNA was digested with *Eco*RI enzyme. Electrophoresis carried out to separate the restriction fragments through 0.8% agarose gels and their transfer to nylon membranes for Southern blot analysis. The whole cosmid was used as a probe, which was labeled with Dig (Boehringer Mannheim Inc.).

1.2.13 Pulse-field gel-electrophoretic analysis of YAC clones

In order to perform pulse-field gel electrophoresis, DNAs were extracted from yeast clones and embedded in agarose plugs as described by Nelson and Brownstein (1994). YAC clones were grown to saturation for about 2 days at 30°C in 50 ml YPD minimal medium lacking uracil and trytophan. Yeast cultures were harvested and resuspended in 0.8 ml of SCEM containing Yeast Lytic enzyme (2 mg/ml), and mixed with 1 ml of 2% low-melting-temperature Seaplaque agarose dissolved in SCE. The hardened plugs were incubated at 37°C for 12 hr to remove the yeast cell walls. Then, these plugs were incubated in 0.5 M EDTA, 10 mM Tris-HCl (pH 8.0), 1% sodium

N-lauroylsarcosine, protease K (0.5 mg/ml) for 16 hrs at 50°C for lysis of the spheroplasts. The plugs were then dialyzed overnight in 10 mM Tris-HCl (pH 8.0), 50 mM EDTA at 4°C and stored in the same solution at 4°C.

YACs were separated from the large yeast chromosomes by pulse-field gel electrophoresis (Hula Gel system, Hoefer Scientific Instruments Inc.) in 1.2% SeaKem GTG agarose gels in 0.5X TBE containing ethidium bromide (1 ug/ml) (Sambrook *et. al.*, 1989) was used as the electrophoresis buffer. Pulse field electrophoresis was performed for 48 hr at 130V and with 110° gel-rotation angles.

1.2.14 Genetic crosses of *N. crassa*

Genetic crosses were performed as described by Davis and de Serres (1970). The female parent was inoculated onto agar slants containing Watergaard's crossing medium and incubated at room temperature for 1 to 2 weeks until protoperithecia were formed. A suspension of fresh conida from the prospective male parent was spread over the protoperithecia and incubated at room temperature for about 2 weeks to mature the asci, which eject their ascospores by light induction. Ascospores were collected using sterile wooden sticks, and transferred to sterile water. The ascospores were activated at 60°C for 60 min and plated on supplemented Vogel's medium and incubated for 12 hr at room temperature until germination was observed. Individual germinated ascospores were collected and transferred to supplemented Vogel's slants. Phenotypic determinations were carried out after conidia were formed by the single-ascospore isolated on the agar slants.

1.2.15 *N. crassa* spheroplast preparation and transformation

The preparation and transformation of *N. crassa* spheroplasts was performed as described previously (Akins and Lambowitz 1985). A suspension of fresh conidia from the appropriate *N. crassa* strain was prepared in sterile water. The suspension was inoculated into 250 ml of Vogel's medium containing appropriate supplements. The final concentration of conidia was about 1.0-1.5 X 10^7 per ml. The culture was incubated with gentle shaking (about 150 rpm) until 90% of conidia were germinated. The conidia were harvested by centrifugation at 5000 rpm for 10 min at 4°C in a Sorvall GS-3 rotor and washed twice with sterile distilled water and once with 1 M sorbitol. The conidial pellet was resuspended in 1 ml of 1 M sorbitol at 1 X 10^7 conidia per ml. The suspension of germinated conidia was incubated with lysing enzyme (3 mg/ml, Sigma Chem. Co.) or Novozym 234 (2 mg/ml, Novo Inc.) at 30°C for 30 min with gentle agitation, to generate protoplasts.

The spheroplast suspension was transferred to a 50-ml screw cap centrifuge tube and centrifuged at the low speed (1000 rpm) in a Sorvall 34 rotor for 10 min. The spheroplasts were washed twice with sterile 1 M sorbitol and once with MCS. Then, spheroplasts were resuspended in MCS as the concentration of 5 X 10^8 per ml. Then, 13 μl of DMSO, 65 μl of sterile heparin (5 mg/ml) and 275 μl of sterile PMC were added to each milliliter of spheroplast solution. This solution was gently mixed and dispensed into sterile Eppendorf tubes to be stored at -80°C

The frozen spheroplasts were thawed on ice and then 1 to 5 µg of DNA in 50 µl dH_2O was added for transformation. The mixture was gently shaken and incubated on ice for 30 min. Then, 9 volume of sterile PMC solution was added to this mixture and incubated at room temperature for 20 min. This mixture was added to pre-warmed 50°C top agar and layered onto proper Vogels agar plates. When the top agar plate was hardened, the plate was incubated in a 4°C cold room for 48 hours. Then, the top agar, which contains hygromicin was layered onto this agar plate. After the top agar has solidified, the plate was incubated at 30°C for 2-5 days to allow colonies to form.

RESULTS AND DISCUSSION

1.3.1 Construction of an *aod-1* expression vector and approach to cloning *aod-2*

The first strategy for cloning *aod-2* was based on the observation that this gene is required for the induction of *aod-1*. Thus, it was assumed that *aod-1*-promoter-driven expression of a bacterial hygromycin resistance gene (*hph*) could be used as a method for selecting *aod-2*$^+$ transformants. For this purpose, a new expression vector, the plasmid (pTAK) was constructed. In this vector, the 6 kb of DNA located upstream of the *aod-1* gene in wild-type *Neurospora* was positioned in the right orientation in front of *hph* in a pBluescriptKS+ vector, which also contains the *N. crassa his-3*$^+$ gene (Figure 1-2). As a negative control, a construct in which the promoter is located in the wrong orientation in front of the *hph* gene also was generated. During the initial phase of creating both constructs, the pCSN43 plasmid (Staben *et al.* 1989) was partially digested with *Cla*I and *Bam*HI to remove the *hph* gene and transcription-termination sequence of the *Aspergillus nidulans trpC* gene. This 2-kb segment of DNA was ligated into pBluescriptKS which had been digested with *Cla*I and *Bam*HI. For the screening of the construct (pHPH), which has the *hph* gene and terminator sequence of *trpC*, double digestions with *Cla*I and *Bam*HI or *Cla*I and *Mlu*I were used. After moving the *hph* and terminator sequence into pBluscriptKS, the *his-3* gene of *N. crassa* was moved into the plasmid. To obtain this construct, the pDV8H plasmid, which contains the *his-3* gene of *N. crassa*, was digested with *Not*I to remove the 2.5 kb *his-3* DNA. This *his-3* DNA was purified from an agarose gel and then ligated into the pHPH construct, which was cleaved with *Not*I. For the screening of the new construct (pHH), *Not*I digestion and hybridization with a probe of *his-3* DNA were used. Then, the pHH plasmid was digested with *Spe*I, blunted by S1 nuclease and religated. SpeI digestion was used for screening the mutant pHH (pHHm) to give uncut DNA. In order to move the putative promoter sequence of the *aod-1* gene, the pHHm was opened with *Cla*I and a 20-bp synthetic adaptor, which contains a *Spe*I site, was inserted. *Spe*I digestion was used for screening the construct(pHHma). Then, the pAOPB-29 plasmid, which contains the promoter of *aod-1* (*Paod-1*) was digested with *Spe*I to remove the 6-kb promoter. This 6-kb DNA was purified from an agarose gel and then ligated in the pHHma plasmid, which was opened with *Spe*I to make the expression vector (pTAK). The digestion with *Spe*I or *Not* I (Figure 1-3) and hybridization were used to screen the DNA construct (data not shown).

The *his-3*$^+$ gene in pTAK serves as a target for the integration of the construct into linkage group I at the *his-3* locus. For this purpose, a *his-3 aod-2* double mutant has been constructed by crossing the appropriate strains. Integration of pTAK into the *his-3* locus of this mutant will generate *his-3*$^+$ autotrophs, which can be selected on minimal medium. We will use these engineered *aod-2 [Paod-1 hph] his-3*$^+$ strain of *N. crassa* to select the *aod-2*$^+$ DNA from 3 cosmid libraries of wild-type *N. crassa* DNA. Protoplasts of *aod-2 [Paod-1 hph] his-3*$^+$ will be transformed with pools of 96 cosmids and transformants will be selected by hygromycin resistance on medium containing oligomycin, which is an inducer of the alternative oxidase. Presumably, spheroplasts that were transformed by a cosmid containing the *aod-2*$^+$ DNA will express the *hph* gene because the *aod-1* promoter has been activated by the Aod-2 protein, which should be induced or activated by oligomycin. Once a group of 96 cosmids that yields *aod-2*$^+$ transformants has been identified, this group will be subdivided into smaller groups. Then, DNAs from the smaller groups will be used to transform the *aod-2 [Paod-1 hph] his-3*$^+$ recipient. The process will be repeated until the cosmid that contains the DNA with the *aod-2*$^+$ allele has been identified. Then the smallest segment of DNA that transforms the *aod-2*$^+$ recipient will be subcloned from the cosmid.

The cloning of *aod-2* by expression of the bacterial *hph* gene from *Paod-1* by inducers of the alternative oxidase in cells that had been transformed by *aod-2*$^+$ DNA proved to be impractical. During the construction of the pTAK, it was discovered that prolonged exposure of the *aod-2* mutant to inducers of the alternative oxidase resulted the expression of *aod-1*. Hence, expression of *hph* occurred in all transformants, regardless of whether or not they received *aod-2*$^+$ DNA. Consequently, this project was abandoned in favor of alternate methods for cloning the *aod-2* gene.

Figure 1-2. Construction of expression vector, pTAK, which contains *his-3*$^{+}$, *hygB*^{r}, and 6 kb upstream region of *aod-1*.

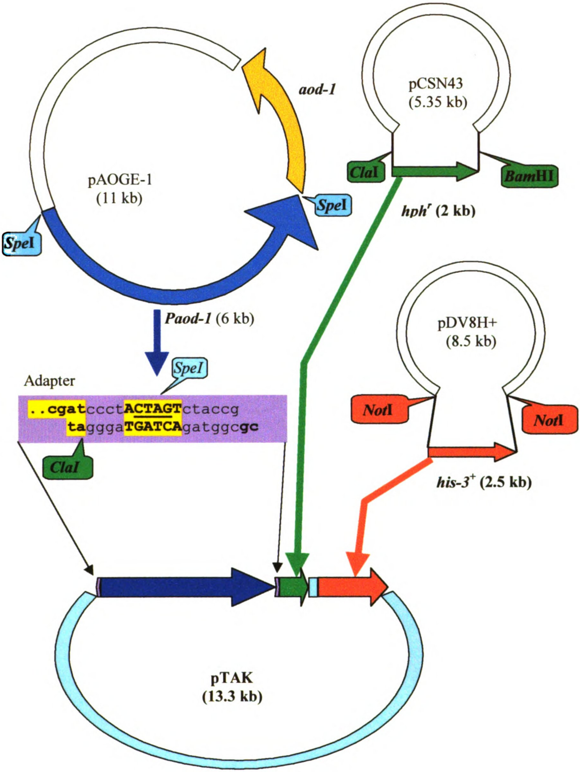
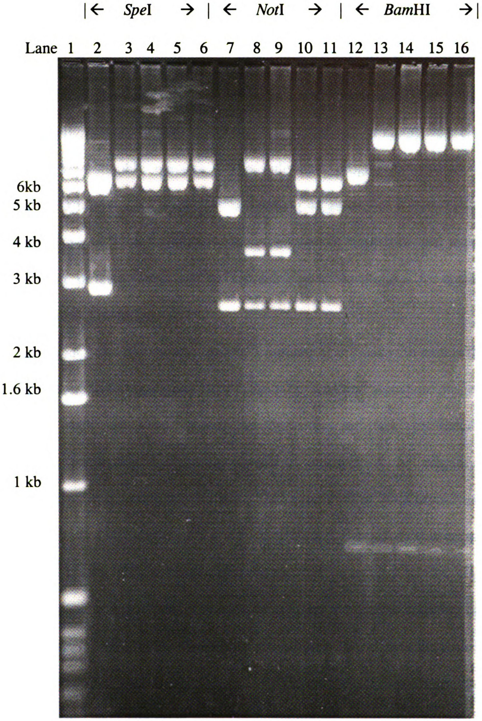


Figure 1-3 Restriction digestion of the pTAK construct to confirm the orientation of each DNA fragment. Lane 1; 1 kb ladder DNA, lanes 2, 3, 4, 5, 6; AO29, pTAK28, pTAK29, pTAK54, pTAK61 digested with SpeI to check the presence of promoter of *aod-1* gene, lanes 7, 8, 9, 10, 11; pHH, pTAK28, pTAK29, pTAK54, pTAK61 digested with NotI to confirm the presence of the *his-3* gene and check the orientation of promoter region of the *aod-1* gene, lanes 12, 13, 14, 15, 16; pHH, pTAK28, pTAK29, pTAK54, pTAK61 digested with *Bam*HI.


|← SpeI →|
← NotI →|
← BamHI →|
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
6kb
5 kb
4 kb
3 kb
2 kb
1.6 kb
1 kb

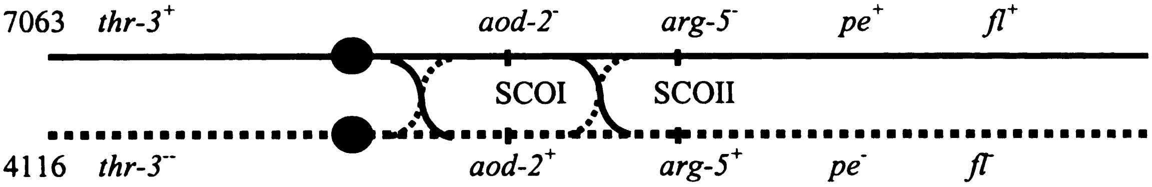
1.3.2 Genetic crosses

In order to generate mutant strains that could be used for the identification of cloned DNAs of the *aod-2* and *arg-5* genes by complementation, several crosses were performed. Initially, strains 7064 (*a aod-2 nic-1 al-3*) and 462 (*A his-3*) were crossed to generate *aod-2 his-3* double mutants. The individual progeny of this cross were identified as TakD followed by a number, and among 45 ascospore isolate TakD 31, 39 and 40 showed the *aod-2 his-3* double mutant phenotype in respiration and requirement tests. These three progeny were kept for screening genomic libraries for cosmids that complement the *aod-2* mutation.

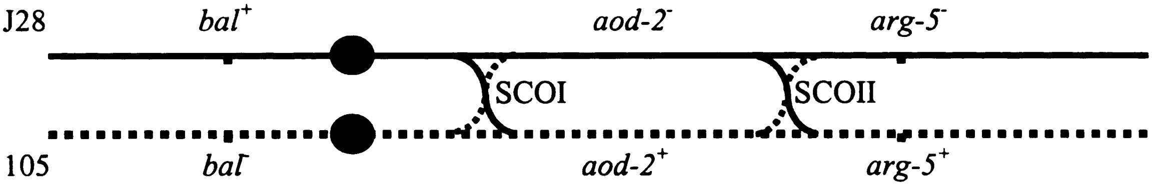
To map *aod-2*, the mutant was used in a number of crosses with strains that have well-defined markers in linkage group IIR (Table 1-1). Random ascospore progeny were collected from each cross and tested for induction or lack of induction of cyanide-resistant respiration by chloramphenicol, antimycin-A or oligomycin, as described in Material and Methods. Initially, 7061 (*aod-2 A*) or 7063 (*aod-2 nic-1 al-3 A*) were crossed with 4116 (*arg-5 pe fl trp-3 a*). J28 (*aod-2 arg-5 A*) was crossed with 105 (*bal a*). The results of these crosses showed that *bal, pe* and *fl* produced high levels of lethality in ascospores, and thus could not be used for the precise mapping of *aod-2*. Consequently a strain, TAKH#15, whose phenotype is *arg-5 thr-3*, was generated from a cross between *arg-5* and *thr-3* strains (Figure 1-4). This strain was crossed with the *aod-2* mutant, and progeny from this cross were tested for the induction of alternative oxidase activity. The results indicated that *aod-2* is located in linkage group IIR, about 3 map units to the left of *arg-5* and 14 map units to the right of *thr-3* (Figure 1-4). To obtain a more precise map position for *aod-2*, crosses were made between J28 (*aod-2 arg-5 a*) and 2102 (*TALS176 A*), which has a translocation (*TALS176*) between the centromere and *arg-5* (Figure 1-5) to check for location of *aod-2* to the left or right of the translocation site (*TALS176*). Due to chromosomal pairing at meiosis, either a 2:1 or a 1:2 (Aod+ Arg+ : Aod- Arg-) phenotypic ratio should appear among the progeny from this cross, depending on the location of the *aod-2* gene to the right or left of the translocation point respectively. The cross yielded 54 progeny of the Aod+ Arg+ phenotype, and 27 progeny with the Aod- Arg- phenotype. Thus, the *aod-2* gene is located to the right of the translocation site, narrowing its position to a location between *TALS176* and *arg-5* in linkage group II (Figures 1-5 and 1-6).

Figure 1-4. Genetic crosses (A) Genetic cross between 7063 (*A aod-2*$^-$ *nic-1*$^-$ *al-2*$^-$) and 4116 (*a arg-5*$^-$ *pe*$^-$ *fl*$^-$ *trp-3*$^-$) strains and genetic map of the region in LGII near the centromere. **(B)** Genetic cross between J 28 (*bal*$^+$ *aod-2*$^-$ *arg-5*$^-$ *a*) and 105 (*bal*$^-$ *aod-2*$^+$*arg-5*$^+$*A*) strains. **(C)** Genetic cross between 7061 (*aod*-2 *A*) and H15 (*arg-5 thr-3 a*). Partial genetic map of Linkage Group II (5 megabases) in *N. crassa*. Genetic mapping of *aod-2* revealed that it is located 3 map units from *arg-5* and 14 map units from *thr-3*. Black dot indicates centromere.

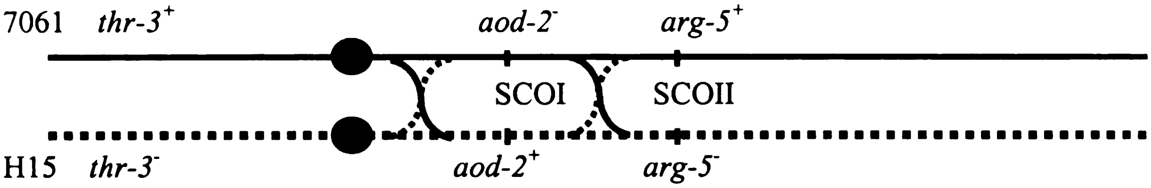
(A) Genetic cross between 7063 (*A aod-2*$^-$ *nic-1*$^-$ *al-2*$^-$) and 4116 (*a arg-5*$^-$ *pe*$^-$ *fl*$^-$ *trp-3*$^-$)



(B) Genetic cross between J 28 (*bal*$^+$ *aod-2*$^-$ *arg-5*$^-$ *a*) and 105 (*bal*$^-$ *aod-2*$^+$*arg-5*$^+$*A*)



(C) Summary of genetic Cross J 7061 (*aod*-2 *A*) × H15 (*arg-5 thr-3 a*).



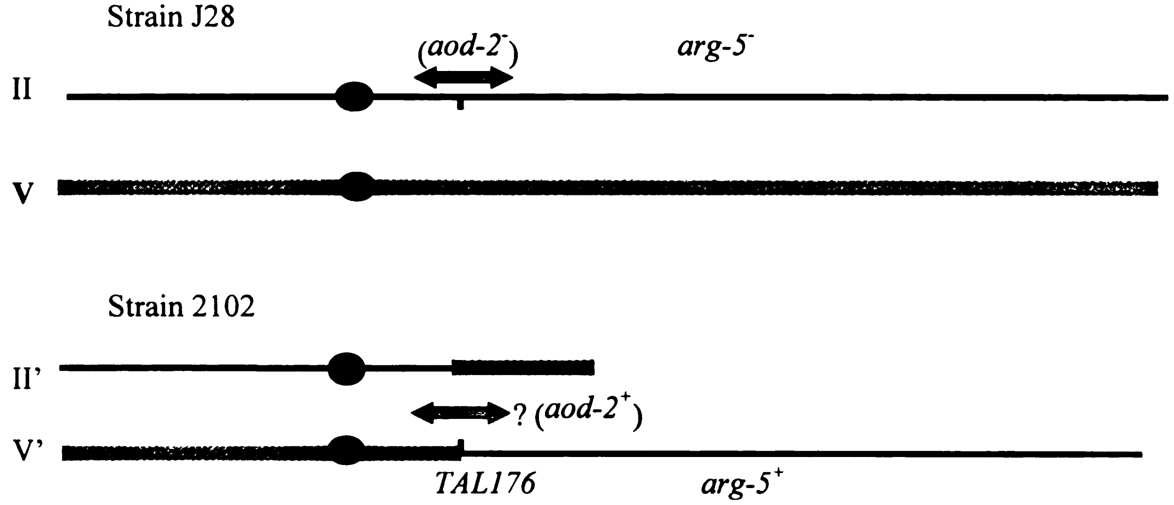
Genotype of progeny	No. of progeny	Frequency	
thr-3 *aod-2*$^+$ *arg-5*	73		
thr-3$^+$ *aod-2* *arg-5*$^+$	66	83.7%	Parental type
thr-3 *aod-2* *arg-5*$^+$	10		
thr-3$^+$ *aod-2*$^+$ *arg-5*	12	13.3%	Single cross over at I
thr-3 *aod-2*$^+$ *arg-5*$^+$	3		
thr-3$^+$ *aod-2* *arg-5*	1	2.4%	Single cross over at II
thr-3 *aod-2* *arg-5*	0		
thr-3$^+$ *aod-2*$^+$ *arg-5*$^+$	1	0.6%	Double cross over I and II
Total	166		

Figure 1-5. Genetic cross between J28 (*a aod-2 arg-5*) and 2102 (*A TALS176*). Yellow arrow indicates the possible location of the *aod-2* gene. The centromere is indicated by a black dot. The translocation site is indicated by a small black line. **(A)** Chromosomal difference between the J28 and 2102 strains. **(B)** Chromosomal pairing at meiosis. **(C)** Possible segregation during meiosis. i) this progeny has a normal LG II and truncated LG V which has a duplication of the right side of LG II. ii) LG II and V come from strain J28 and will show same phenotype as the parent. iii) LG II and V with translocations and this progeny show the same phenotype as strain 2102. iv) normal LG V and most of LG II is missing. This condition is lethal and not show in the progeny.

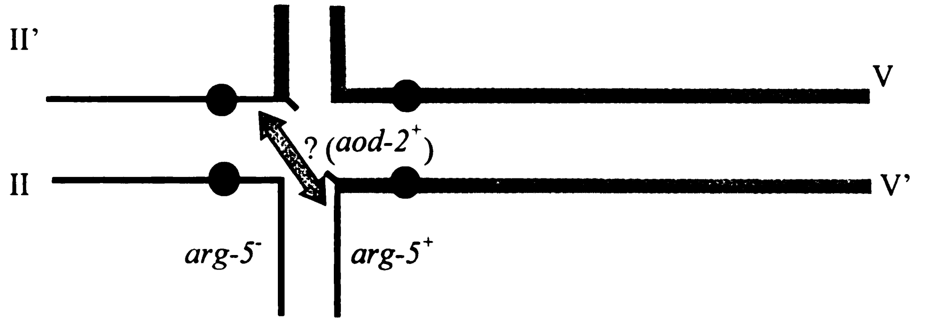
Phenotype	Number of progeny	%	Expected ratio (I)	Expected ratio (II)
aod+ arg+	54	55	1/3	2/3
aod+ arg-	1	1	0	0
aod- arg+	2	2	0	0
aod- arg-	27	27.5	2/3	1/3
Total	98			

I: if the *aod-2* gene is on the left side of the translocation site, II: if the aod-2 gene is on the right side of the translocation site.

(A) Chromosomal difference between J28 and 2102 strains.



(B) Chromosomal pairing at meiosis.



(C) Possible segregation during meiosis

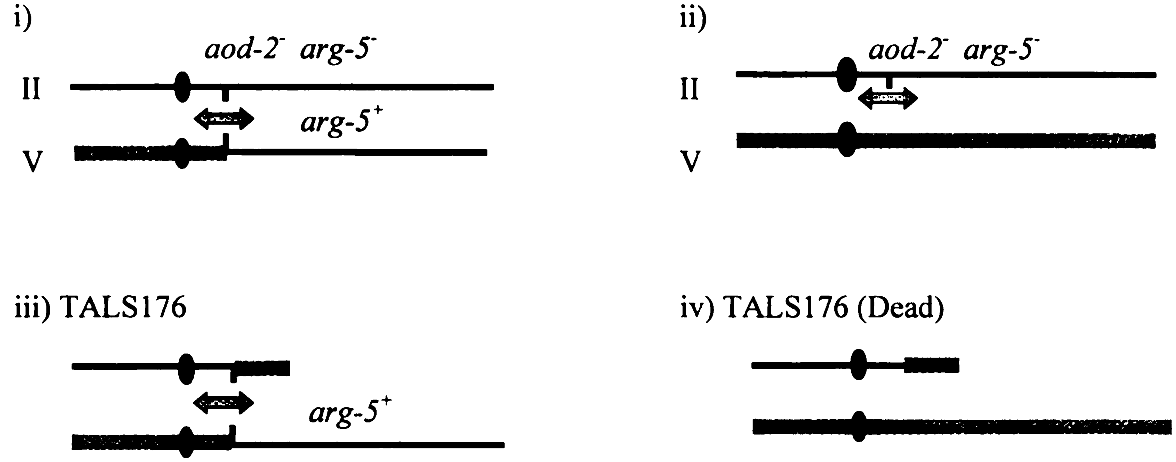
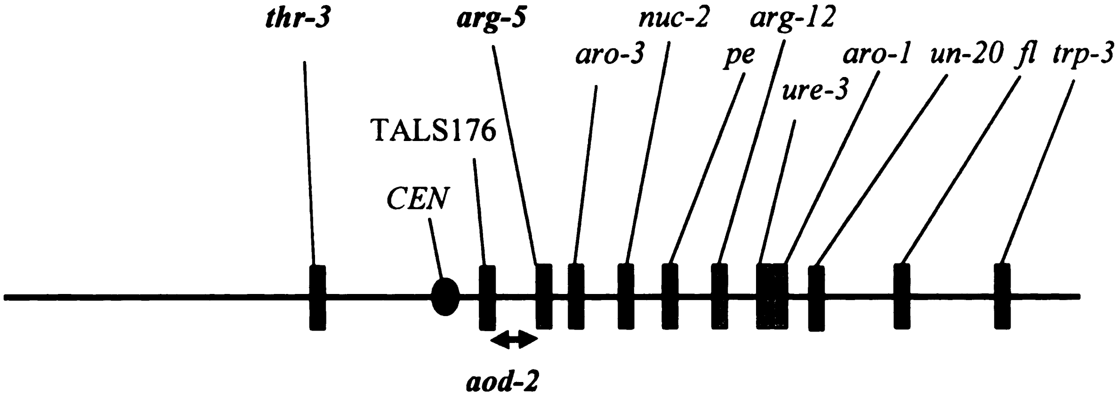


Figure 1-6. Partial genetic map of Linkage Group II (5 megabases) in *N. crassa*.



1.3.3 Chromosome walking

Chromosome walking is another strategy for identifying the *aod-2* gene. This requires the determination of a reasonably precise map position for *aod-2* and identification of a very well defined marker DNA which is close to *aod-2* in linkage group II. In this study we used the *arg-5* gene as a marker and two known cosmids, G8:11H from the pMOCosX library and 25:1D from the pSV50 library, as the starting points for a chromosomal walk. The G8:11H cosmid was digested with *Eco*RI, and T3 or T7 primers used to generate the end-specific RNA probes. These probes were hybridized to the 50 plate-pool DNAs of the pMOcosX cosmid library as described in Material and Methods. The results of the hybridizations revealed that the G7, G8, X10, X12, and X25 plate pools were detected by the T3 probe of G8:11H, and the G8 plate pool gave the strongest signal (Figure 1-7). The T7 probe indicated that G3, G7, G8, X10, X12, and X25 plate pools contained cosmids that overlap G8:11H (Figure 1-8). The row and column plate-pool DNAs from the X25 plate were prepared, cut with *Eco*RI and electrophoresed on an agarose gel as described in Materials and Methods. The hybridization showed that the X25:7A cosmid was the next on the contig (Figure 1-9). The result of the hybridization with the T7 probe of X25:7A cosmid showed that the G2, G7, X10, X2, X3, X10, X16, X17, and X25 plate-pool DNAs were hybridized (data not shown). The T3 probe of X25:7A cosmid identified plates G3, G7, G8, G11, X10, X12, X22, X25. Plate X10 was selected and the row and column pool DNAs were prepared and separated by electrophoresis on an agarose gel. Cosmid X10:12G was identified by both the T3 and T7 probes of the X25:7A cosmid. The T3 end-probe of the X10:12G cosmid gave strong hybridization with X25:7A and the G8:11H cosmids. However, the T7 end-probe of X10:12G hybridized with X25:7A, but not with G8:11H. Thus, the cosmid X10:12G yielded a small extension of the contig from G8:11H. Nonetheless, the hybridization experiment with T3 and T7 probes of X10:12G did not yield a new plate-pool of DNAs that could be used to extend the walk. Thus, the X2 plate was selected for further chromosomal walking from the cosmid X25:7A. The row and column pools of DNAs of the X2 plate revealed that cosmid X2:7C hybridized with the T7 probe of X25:7A but not with the T3 probe. Both the T3 and T7 end-probes of X10:12G did not hybridize with any row and column pools of DNAs from the X2 plate. The T3 end probe of X25:7A strongly hybridized with cosmid G8:11H but the T7 end-probe did not hybridize with this cosmid. Thus, the T7 end probe of X25:7A was used to extend the contig by screening the plate-pool DNAs. The hybidization experiment showed that G2, G7, G8, X2, X3, X10, X12, X16, X17, X22, and X25 plate-pool DNAs hybridized with the T7 probe of X25:7A. The hybridization with the cosmids of the contig revealed that cosmid 25:1D (pSV50 cosmid library) was recognized by the T3 probe of X2:7C but not by the T7 end-probe of X2:7C. Thus, 3 cosmids (X10:12G, X25:7A, and X2:7C) fill the gap between the cosmids G8:11H and 25:1D. The G and X plate pools DNAs were screened with the T3 and T7 probes from the X2:7C cosmid and the T3 probe of X2:7C pulled out G1, G2, G3, G7, G8, X1, X2, X3, X4, X6, X8, X10, X17, X22, and X24 plate pools. Another cosmid was pulled out by probes from the X25:7A cosmid. Both the T3 and T7 probes of X25:7A hybridized with the G7:3D cosmid, but only T3 and not the T7 probe of X10:12G hybridized with G7:3D. Thus, the G7:3D cosmid extended the contig further from the X10:12G cosmid. Hybridization experiments with probes from this

G7:3D cosmid revealed that the T7 probe did not hybridize with X2:7C but hybridized with the G8:11H, X25:7A, and X10:12G cosmids. However, the T3 probe of G7:3D cosmid hybridized with all cosmids (X2:7C, X25:7A, X10:12G, and G8:11H) in this contig except 25:1D. Screening G and X plate pool DNAs with the end probes of G7:3D showed that G3, G7, G8, X10, X12, X22, X25 plates were detected by the T3 probe, whereas the T7 probe detected G7, G8, X10, X12, X25 plate-pool DNAs. Extension of the contig with the T7 probe of G7:3D identified the X12:5D cosmid. From the X12:5D cosmid, G3, G7, G8, X6, X8, X10, X12, X24, and X25 plate-pools were identified by using the T3 probe, whereas the T7 end probe identified the G3, X10, X12, and X25 plate-pools. RNA probes could not be generated from the 25:1D cosmid with T3 or T7 primers. Thus, this cosmid was digested with *Eco*RI and 7.5 kb, 6 kb, 5 kb, and 3.8 kb DNA bands were purified from an agarose gel. Probes were made from the purified restriction fragments as described in Materials and Methods. A hybridization experiment with the 7.5-kb probe pulled out G1, X10, X12, and X24 plate-pools, and the screening of G1 row and column pool DNAs showed that G1:1C and G1:11H were recognized. The T3 probe of the G1:1C cosmid identified a new plate-pool DNA, G3, but the T7 probe did not recognized any plate-pool DNAs. The T7 probe of G1:11H hybridized specifically with the X24:12B cosmid. However, RFLP mapping revealed that the X24:12B cosmid is located near the centromere of the linkage group I, and not in the linkage group II (data not shown).

A partial sequence was obtained from the T3 end of cosmid X2:7C and 2 sets of primers, set1 (HB337 and HB338) and set 2 (HB385 and HB386), were designed to identify the YAC clone by PCR (Figure 1-10). The PCR reaction with set 1 primers did not amplify any of the genomic DNA of *N. crassa*, but a 190-bp long fragment was amplified with primer set 2 from the X2:7C cosmid and *N. crassa* chromosomal DNA templates. Thus, the HB386 and HB387 primers were used for screening YAC plate-pool DNAs, and YAC 2:6D was identified to overlap cosmid X2:7C. Pulse-field gel electrophoresis was performed to purify the YAC DNA as described in Materials and Methods (Figure 1-11). A hybridization experiment was performed to identify the portion of the YAC (Figure 1-12) that overlapped with the established contig. The purified YAC DNA was used to make end-specific probes by using the NL and NR primers as described in Material and Methods. Screening of the cosmid library was continued with the NL and NR probes by Southern hybridization. The G3:10C cosmid was identified by the NL probe, but the NR probe did not identify a new cosmid (Figure 1-13) in the library.

The chromosome walk has generated a contig which consists of 10 cosmids and one YAC (Figure 1-14). We know that the genomic DNAs in 2 cosmids (25:1D in the Vollmer and Yanofsky library, and G8:11H in the Orbach/Sachs library) are located close to the *arg-5* gene (overlapping or similar RFLP patterns). These were used as starting points for a chromosome walk to *aod-2* by hybridization with other cosmids. The 10 neighboring cosmids were used to transform spheroplasts of the *arg-5 aod-2* mutant strain J28. Transformants were selected by growth on media containing either benomyl or hygromycin, depending on the selectable marker provided by the cosmid. To determine whether or not the transformants that had received *aod-2*$^+$ DNA, respiration tests were performed to see if the alternative oxidase was induced by oligomycin. However, the complementation tests showed that none of the cosmids on the contig

(G3:10C, G1:11H, G1:1C, 25:1D, X2:7C, X25:7A, X10:12G, G8:11H, G7:3D, X12:5D) did complemented either the *aod-2* or *arg-5* defects.

Figure 1-7. Southern hybridization screening pMOcosX cosmid library G and X plate pool DNAs with T3 end RNA probe of G8:11H cosmid. All plate pool DNAs were digested with *Eco*RI enzyme. Lanes 1, pSV50; 2, G811H; 3, 1 kb ladder DNA; 4 through 20, G plates #1 through 17. Lanes 21, pSV50; 22, G811H; 23, 1 kb ladder DNA; lanes 24 through 40 represent X plate pool DNAs #1 through 17. Lanes 41 through 47 represent to G plate pool DNAs #18 through 25; and lane 48, 1 kb ladder DNA; 49, pSV50; 50, G811H; 51, 1kb ladder DNA; lanes 53 through 60 represent X plate pool DNAs #18 through 25.

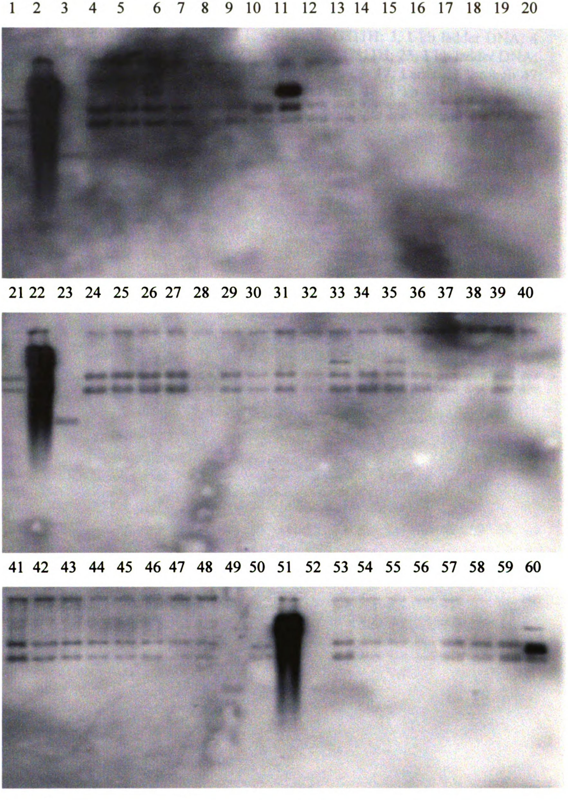
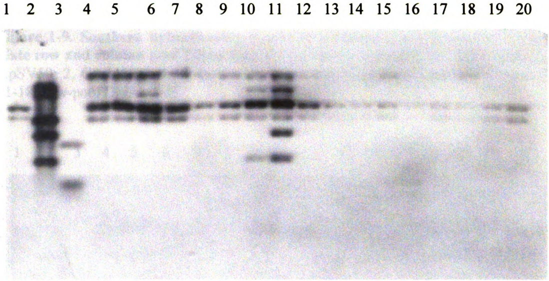
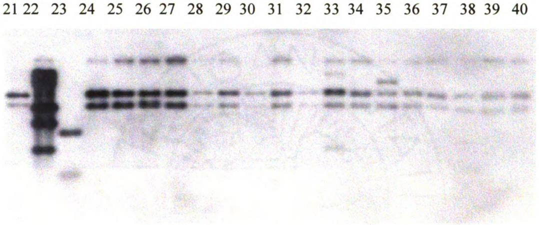

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Figure 1-8. Southern hybridization screening pMOcosX cosmid library G and X plate pool DNAs with T7 end RNA probe of G8:11H cosmid. All plate pool DNAs were digested with *Eco*RI enzyme. Lanes 1, pSV50; 2, G811H; 3, 1 kb ladder DNA; 4 through 20, G plates #1 through 17. Lanes 21, pSV50; 22, G811H; 23, 1 kb ladder DNA; lanes 24 through 40 represent X plate pool DNAs #1 through 17. Lanes 41 through 47 represent to G plate pool DNAs #18 through 25; and lane 48, 1 kb ladder DNA; 49, pSV50; 50, G811H; 51, 1kb ladder DNA; lanes 53 through 60 represent X plate pool DNAs #18 through 25.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20


21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

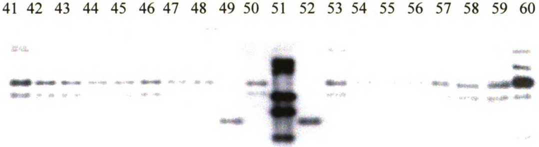

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Figure 1-9. Southern hybridization screening pMOcosX cosmid library X25b half plate row and column pool DNAs with T7 side RNA probes of G8:11H cosmid. Lane 1,pSV50; 2, G8:11H; 3, 1 kb ladder DNA; lanes 4-12, column pool DNAs 7-12; lanes 11-18, row-pool DNAs A-H.

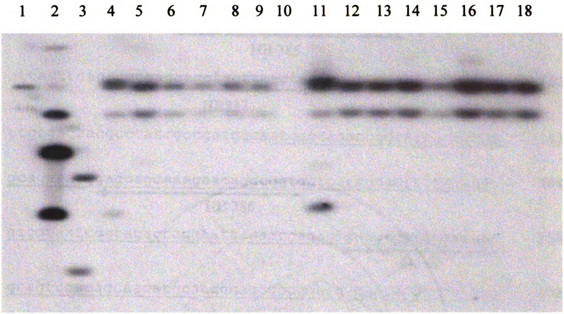


Figure 1-10. Partial sequence of the T3 end of cosmid X2:7C and locations of primers used to identify a YAC clone. The underlined sequence is ORF for unknown.

ccccagccctcttgctt**ggctttcttctcccgttccttctc**catcttgtgc 51

HB 385

atcacttcagccgt**gagctcctgcttcagtaa**tcgctccaagacatccttc 102

HB337

ttgcgagtacgcctatcgccgatggcaatgagcaaaccggcagtactgttg 153

gcagaaatg**gagcagcaaagaacaggcggtg**gtcactgcacttttcctggt 204

HB 386

gtggtaatcggtagaccaggtataagaacgagag**tgatctggagacactgt** 255

HB338

gcagtccacagcaacatgcatggcagcggcggtggtcactgcacttttctg 306

tgtgtatcgagaccg 357

Figure 1-11. Pulse-field gel electrophoresis of YAC 2:6D. Lanes 1 and 3 are wild type yeast and lanes 2 and 4 through 20 are YAC 2:6D. The arrow indicates YAC.

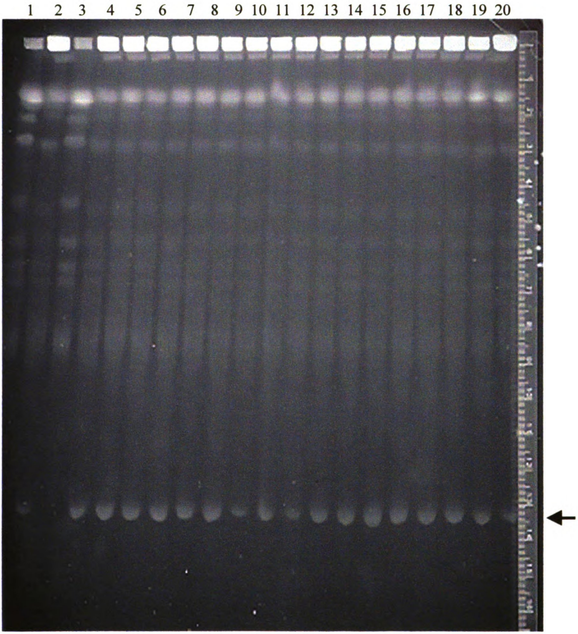


Figure 1-12. Southern hybridization of YAC 2:6D with identified cosmids. Lane 1, hybridization result with T3 end probe from X24:12B; 2, hybridization result with T7 G1:11H probe; 3, hybridization result with T3 G1:11H probe; 4, hybridization result with T3 G1:1C probe; 5, hybridization result with T7 G1:1C probe; 6, hybridization result with 25:1D probe; 7, hybridization result with T3 X2:7C probe; 8, T7 X2:7C probe; lane 9, hybridization result with T7 side probe of G7:3D; 10, hybridization result with T3 G7:3D probe; 11, hybridization result with T3 G8:11H probe; 12, hybridization result with T3 G8:11H probe; 13, hybridization result with T7 X10:12G probe; 14, hybridization result with T3 X10:12G probe; 15, hybridization result with T7 X25:7A probe; 16, T3 X25:7A probe.

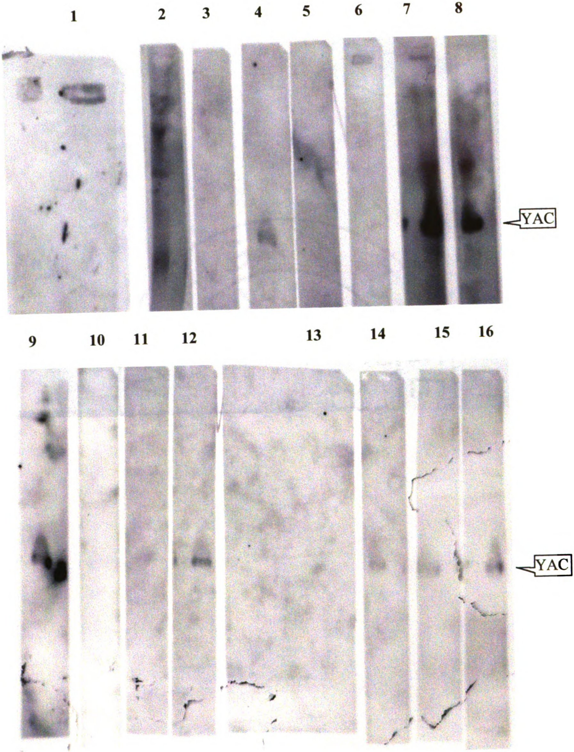
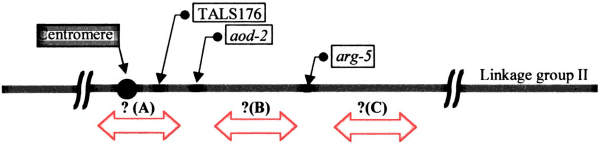
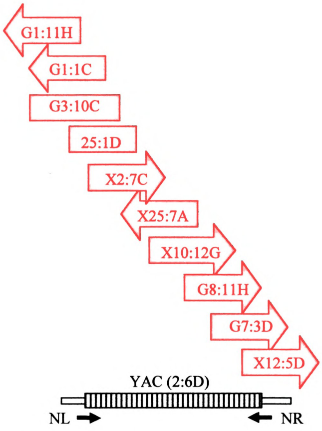

1
2
3
4
5
6
7
8
YAC
9
10
11
12
13
14
15
16
YAC

Figure 1-13. Southern hybridization screening pMOcosX cosmid library G3 plate row and column plate pool DNAs with single-strand DNA NL probe of YAC 2:6D.

Figure 1-14. Chromosomal walking in LG II near *arg-5*. Arrow indicates T7 site of cosmid. The contig could be localized in one of 3 regions: **A**, which is to the left side of *aod-2*; **B** which is located between *aod-2* and *arg-5*; and **C**, which is located to the right side of *arg-5*.



Contig



NL primer 5'-GAAGAAAGAGTATTACTACATAAC

NR primer 5'-CATTCACTTCCCAGACTTGC

1.3.4 Attempted cloning of the *arg-5* gene using degenerate primers

An alignment of acetylornithine aminotransferase from *Alnus glutinosa* (Y08680), *Synechocystis sp.* (D90904), *E. coli* (M32796), *Saccharomyces cerevisiae* (M32795) is shown in Figure 1-12 and revealed two highly conserved regions that were selected to generate degenerate primers for amplification of a portion of the *N. crassa arg-5* gene by PCR from genomic DNA. The distance between the two degenerate primers was anticipated to be less than 300 bp (Figure 1-15). An initial PCR reaction using these primers resulted in a product of the expected size (~300 bp, data not shown). Various conditions were used to amplify the internal region of the *arg-5* gene of *N. crassa*. However, the reaction did not yield an obvious PCR product from genomic DNA (Figure 1-16). Thus, the three sets of DNA fragments were purified from an agarose gel to generate Dig-labelled probes, which where used to screen cosmid libraries of *N. crassa* genomic DNA. The probe generated from band A did not hybridize distinctly with any cosmid in the library, whereas the probe generated from band C hybridized with a large number of different cosmids. In contrast the probe generated from band B hybridized strongly with a single cosmid, G5:2D. However, transformation revealed that this cosmid did not complement the *arg-5* mutation. Moreover, RFLP mapping showed that this cosmid probably is located between *vma-1* and *al-3* in the LG V (Figure 1-17), rather than on LGII. Hence, *arg-5* could not be used as an anchor for the chromosome walk to *aod-2*.

Figure 1-15. Generation of degenerate primers for the isolation of the *arg-5* DNA from *N. crassa*. **A)** Multiple alignment of acetylornithine aminotransferase sequences. Amino acid-sequences were aligned by the clustal method of the DNASTAR program. The highly conserved residues are shown in the green shaded boxes. Identical residues are marked with asterisks above the sequences. The location of degenerate primers is indicated by red arrows above the sequence. Ag, *Alnus glutinosa* (Y08680); Sy, *Synechocystis sp.* (D90904); Ec, *E. coli* (M32796); Sc, *Saccharomyces cerevisiae* (M32795). **B)** Sequence of 5'and 3' degenerate primers. Abbreviations represent the following combinations of nucleotides: N=A, C, G, or T; Y=C or T; I=deoxyinosine; R=A or G. The standard one letter code is used for the amino acids.

A)

```
        *                                                              *
Ag      MTSLQYFSLNRPVFPATHLHRPGIRHLQVSACANVEVQAPSSVKKQGVSKEVMEAAGRVLVGTYARVP-V  69
Sy      MT-----------------YSPVVESVEAQAFAVTDLSPAAEFKTADFDTYVMN--------TYGRFP-I  44
Ec      MS--------------------------------------QPITRENFDEW-MIP-------VYAPAP-F  23
Sc      M------------------FKRYLSSTSSRRFTSI-------LEEKAFQ-----------VTTYSRPEDL  34

           **      *   *  * *   ***   ****          *     *  *
Ag      VLSRGKGCKLYDP-EGREYLDLSAGIAVNVLGHADSDWLRAVTEQAATLTHVSNVFYSIPQVELAKRLVA 138
Sy      AIARGQGSTLWDT-EGKSYLDFVAGIATCTLGHAHPALVRAVSDQIQKLHHVSNLYYIPEQGELAKWIVE 113
Ec      IPVRGEGSRLWDQ-QGKEYIDFAGGIAVNALGHAHPELREALNEQASKFWHTGNGYTNEPVLRLAKKLID  92
Sc      CITRGKNAKLYDDVNGKEYIDFTAGIAVTALGHANPKVAEILHHQANKLVHSSNLYFTKECLDLSEKIVE 104

                                             r (HB412)
                  * ***  *** ****** *   *                       *****
Ag      SSF-------ADRVFFSNSGTEANEAAIKFARKFQRFTRPDEKQPATE-FVSFSNSFHGRTMGSLALTSK 200
Sy      HSC-------ADRVFFCNSGAEANEAAIKLVRKYAHTVLDFLEQPV---ILTAKASFHGRTLATITATGQ 173
Ec      ATF-------ADRVFFCNSGAEANEAALKLARKFAHDRYG-SHKSG---IVAFKNAFHGRTLFTVSAGGQ 151
Sc      KTKQFGGQHDASRVFLCNSGTEANEAALKFAKK-----HGIMKNPSKQGIVAFENSFHGRTMGALSVTWN 169

                                                                     r (HB 413)
          *   *                                     ** ******              *
Ag      ENYRSPFEPVMPGVTFLEYGNIEAATQLIQ-----RRKIAAVFVEPIQGEGGVYSATKEFLYALRKACDD 265
Sy      PKYQQYFDPLVPGFDYVPYNDIRSLENKVADLDEGNSRVAAIFLEPLQGEGGVRPGDLAYFKRVREICDQ 243
Ec      PAYSQDFAPLPADIRHAAYNDINSASALIDD------STCAVIVEPIQGEGGVVPASNAFLQGLRELCNR 215
Sc      SKYRTPFGDLVPHVSFLNLND--EMTKLQSYIETKKDEIAGLIVEPIQGEGGVFPVEVEKLTGLKKICQD 237

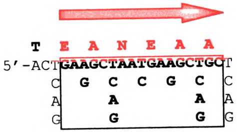
               **** * **   *           **  * ** *  * * **             * *  *
Ag      SGTLLVFDEVQCGLGRTGYLWAHEIY--DVFPDIMTLAKPLAGGLPIGAVLVTERVASAITYGDHGTTFA 333
Sy      NDILLVFDEVQVGVGRTGKLWGYEHL--GVEPDIFTSAKGLAGGVPIGAMMCKKFCD-VFEPGNHASTFG 310
Ec      HNALLIFDEVQTGVGRTGELYAYMHY--GVTPDLLTTAKALGGGFPVGALLATEECARVMTVGTHGTTYG 283
Sc      NDVIVIHDEIQCGLGRSGKLWAHAYLPSEAHPDIFTSAKALGNGFPIAATIVNEKVNNALRVGDHGTTYG 307

        * **       **  *     *  *          *             **  **  *
Ag      GGPLVCKAALTVLDKILRPGFLASVSKKGHYFKEMLINKLGGNSHV-REVRGVGLIVGIELD----VSAS 398
Sy      GNPLACAAGLAVLKTIEGDRLLDNVQARGEQLRSGLAEIKNQYPTLFTEVRGWGLINGLEISAESSLTSV 380
Ec      GNPLASAVAGKVLELINTPEMLNGVKQRHDWFVERLNTINHRYG-LFSEVRGLGLLIGCVLNADYAGQAK 352
Sc      GNPLACSVSNYVLDTIADEAFLKQVSKKSDILQKRLREIQAKYPNQIKTIRGKGLMLG----AEFVEPPT 373

                 *     **   * *  * *
Ag      PLVNACLNSGLLVLTAGKGNVVRIVPPLIITEQELEKAAEILLQCLPALDRHG                  451
Sy      EIVKAAMEQGLLLAPAG-PKVLRFVPPLVVTEAEIAQAVEILRQAIATLV                     429
Ec      QISQEAAKAGVMVLIAG-GNVVRFAPALNVSEEEVTTGLDRFAAACEHFVSRGSS                389
Sc      EVIKKARELGLLIITAGKSTV-RFVPALTIEDELIEEGMDAFEKAIEAVY----A                423
```

B) Degenerate primers

5'region primer **5'-GARGCIAAYGRGCIGC** : 17 mer (HB412)



3'Region Primer **5'-ACICCICCYTCICCYTG:** 17 mer (HB413)

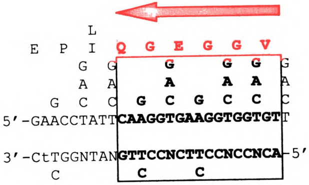


Figure 1-16. PCR amplification of acetylornithine aminotransferase using degenerate primers. Left lane contains 100 bp ladder DNA and the right lane showed the PCR products obtained by PCR with degenerate primers. Three (Band A, B and C) sets of DNA fragments were purified from agarose gel and used to make probes.

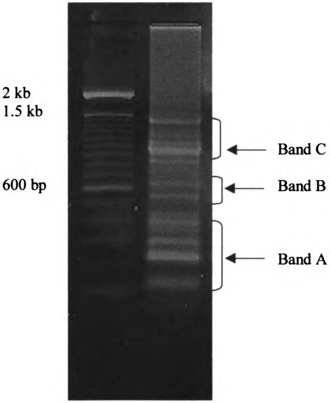


Figure 1-17. Restriction Fragment Length Polymorphism (RFLP) mapping of the G5:2D cosmid. Genomic DNAs were isolated from the *N. crassa* strain Mauriceville, and Oak Ridge #4450–4488 (Fungal Genetics Stock Center) strainsand 5 μg of each DNA was digested with *Eco*RI, electrophoresed on an agarose gel and transferred to a nylon membrane. Cosmid G5:2D, was labeled with Dig (Boehringer Mannheim Inc.) and used as a probe. The restriction pattern in each lane is designated as O (Oak Ridge) or M (Mauriceville). The location of the cosmid G5:2D is probably between *vma-1* and *al-3* in LG V.

LG V

	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87
	A	A	B	B	C	C	D	D	E	E	E	E	F	F	G	G	H	H	I	I	J	J	K	K	L	L	M	M	N	N	O	O	P	P	Q	Q	R	R
	1	4	6	7	1	4	5	7	1	3	5	7	1	3	1	4	5	7	6	8	1	4	1	4	1	4	5	8	2	3	2	4	1	4	2	4	1	4
AP8w.4	O	M	M	O	M	O	M	O	M	O	M	O	M	M	M	O	M	O	O	M	M	O	M	M	M	M	M	O	O	O	M	M	O	M	M	M	M	M
rDNA, X15:4E, AP5c.2	O	M	M	O	M	O	M	O	M	O	M	O	M	M	M	O	M	O	O	M	M	O	M	M	M	O	M	O	O	O	M	M	O	M	M	M	O	M
AP34c.3, R44.3	O	M	M	O	M	O	M	O	M	O	M	O	M	-	M	O	M	O	O	M	M	O	M	-	M	O	M	O	O	-	M	M	O	M	M	M	O	M
AP31a.8, R15.8	O	M	M	O	M	O	M	O	M	O	M	O	M	M	M	O	M	O	O	O	M	O	M	M	M	O	M	O	O	O	M	M	O	M	M	M	O	O
AP5c.3	O	M	O	O	O	O	M	O	M	O	M	O	M	M	O	O	M	-	O	M	M	O	M	M	M	O	M	O	O	O	M	M	O	M	M	M	O	M
AP13.3	O	M	O	O	O	O	M	O	M	O	M	O	M	M	O	O	M	O	O	M	M	O	M	M	M	O	M	O	O	O	M	M	O	M	M	M	O	O
con-2	O	M	O	O	O	O	O	O	M	O	M	O	M	M	O	O	M	O	O	M	M	O	M	M	M	O	M	O	O	O	M	M	O	M	M	M	O	O
AP4a.6	O	M	O	O	O	O	O	O	O	O	M	M	M	M	O	O	M	M	O	M	M	M	M	M	O	O	M	O	O	O	M	M	M	M	M	M	O	O
pSK21d	M	M	O	O	O	O	O	O	M	O	M	M	M	M	O	O	M	M	O	O	M	M	M	M	O	O	M	M	O	O	M	M	-	M	M	M	O	-
AP5c.4	M	M	O	O	O	O	O	O	O	O	M	M	O	M	O	O	M	M	O	O	M	M	M	M	O	O	M	M	O	O	M	M	M	O	M	M	O	O
Fsr-16	M	M	O	O	O	M	O	O	O	O	M	M	-	M	O	O	M	M	-	-	-	-	M	M	O	O	M	M	O	-	M	M	M	-	-	-	-	-
Fsr-9	M	M	O	O	O	M	O	O	O	O	M	M	M	M	O	O	M	M	O	O	M	M	M	M	O	O	M	M	O	O	M	M	M	M	M	M	O	O
Cen V, lys-1, ccg-8	M	M	O	O	O	O	O	O	O	O	M	M	M	M	O	O	M	M	O	O	M	M	M	M	O	O	M	M	O	O	M	M	M	M	M	M	O	O
AP36c.2, R64.2	M	M	O	O	O	O	O	O	O	O	M	M	M	O	O	O	M	M	O	O	M	M	M	O	O	O	M	M	O	O	M	M	M	M	M	M	M	O
AP12g.1	M	O	O	O	O	O	O	O	O	O	M	M	M	O	O	O	M	M	O	O	M	M	M	O	O	O	M	M	O	M	M	M	M	M	O	M	M	O
ccg-1=grg-1	M	O	O	O	M	O	O	O	O	O	M	M	M	O	O	O	M	M	O	O	M	M	M	O	O	O	M	M	O	M	M	M	M	M	O	M	M	O
X11:D2	M	O	O	O	O	O	O	O	M	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	O	M	M	O	M	O	M	M	M	M	M	M	O
AP8w.7	M	O	O	O	O	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	O	M	M	O	M	O	M	M	M	O	M	M	O
leu-5	M	O	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	O	M	M	O	-	O	M	M	M	O	M	-	O
mfa	M	O	O	O	M	O	O	M	O	-	-	M	M	O	O	O	M	M	M	O	O	M	M	O	O	O	M	M	O	M	O	M	M	M	O	M	M	O
ilv-2	-	O	-	-	-	-	O	O	-	-	-	-	-	-	-	-	-	M	O	O	M	-	M	O	-	O	-	M	O	-	O	-	-	M	M	M	M	O
nuo24	M	-	O	-	M	O	O	M	O	O	M	M	M	O	O	O	-	M	O	O	-	M	M	O	O	M	-	M	O	M	O	M	M	M	O	M	M	-
tom40	-	O	-	-	M	O	O	M	O	O	M	M	M	-	O	O	M	M	O	O	O	-	-	-	O	M	M	M	O	M	O	-	-	-	O	M	M	O
cyh-2, MnSOD, 14:10G, mc	M	O	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	M	O	M	M	M	O	M	M	O
rca-1	M	O	O	O	M	-	O	M	-	-	-	-	M	O	O	O	M	M	-	O	O	M	M	O	-	-	-	-	O	M	O	M	M	M	O	M	M	O
G16:11H	M	O	O	O	M	O	O	M	O	-	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	M	O	M	M	M	O	M	M	O
X23:C8	M	O	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	-	O	M	M	M	O	M	M	O
vma-1, vma-3	M	O	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	-	O	M	M	-	-	-	M	-	-	M	O	M	M	M	O	-	M	O
G5:2D	**M**	**O**	**O**	**-**	**M**	**O**	**O**	**O**	**O**	**O**	**M**	**M**	**-**	**-**	**O**	**M**	**M**	**M**	**O**	**O**	**M**	**M**	**M**	**-**	**M**	**M**	**M**	**M**	**O**	**M**	**O**	**M**	**M**	**M**	**O**	**M**	**M**	**O**
23:1A	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	M	O	M	M	M	O	M	M	O
18:10A	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	-	M	M	M	-	M	-	M	M	M	O	M	M	O
AP9b.1	-	M	-	O	M	O	O	O	O	O	-	-	M	O	O	O	M	M	O	O	O	M	-	O	O	M	-	-	O	-	O	-	-	-	M	-	M	O
al-3	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	M	O	M	M	M	O	M	M	O
ccg-6	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	M	M	M	M	M	O	M	M	O
AP31b.2, R23.2	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	O	O	M	M	O	O	M	M	M	O	M	-	M	M	-	O	M	M	O
AP3b.3	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	-	M	M	O	O	-	M	M	-	O	M	M	M	O	M	O	M	M	M	O	M	M	O
inl	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	M	O	M	M	O	O	M	M	M	O	M	O	M	M	M	O	M	M	O
cit-2	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	-	M	O	M	M	M	O	M	M	M	O	M	O	M	M	M	O	M	M	O
cya-2	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	O	M	M	O	M	O	M	O	M	O	M	M	M	O	M	O	M	M	M	O	M	M	O
hspe-1	M	M	O	O	O	O	O	M	O	O	M	M	M	O	O	M	M	M	O	M	O	M	O	M	O	M	M	M	M	M	O	M	M	M	O	M	M	O
cmd	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	M	M	M	-	M	O	M	O	M	O	M	M	M	M	M	O	M	M	M	O	M	M	O
hsp83	M	M	O	O	M	O	O	M	O	O	M	M	M	O	O	M	M	M	O	M	O	M	O	M	O	M	M	M	M	M	O	M	M	M	O	M	M	O
tom70	-	M	-	O	M	O	-	M	O	O	M	M	M	O	O	M	M	M	O	M	O	M	M	M	O	M	M	M	M	M	-	-	M	M	O	M	M	O
Fsr-20	O	M	O	O	M	M	O	M	O	O	M	M	M	M	M	M	M	M	O	M	O	M	M	M	O	M	M	M	M	-	O	M	M	O	O	M	M	O
AP34b.3, R42.3	O	M	O	O	M	O	O	M	O	O	M	M	M	M	O	M	M	M	O	M	O	M	M	M	O	M	M	M	M	M	O	M	M	O	O	M	O	O
8:9A	O	M	O	O	M	O	O	M	O	O	M	M	M	M	M	M	-	O	O	M	O	M	M	M	O	M	M	M	M	M	O	M	M	O	O	M	M	O
crp-4	O	M	O	O	O	O	O	M	O	-	M	M	M	M	M	M	M	O	O	M	O	M	M	M	M	M	M	M	M	M	O	M	-	O	O	M	M	O
clone 129	O	M	O	M	M	O	O	M	O	O	M	M	M	M	M	M	M	O	O	M	O	M	M	M	O	M	M	M	M	M	O	M	M	O	O	M	M	O
AP16.2	O	M	O	M	M	O	O	M	O	O	M	M	M	M	M	M	M	O	O	M	O	M	M	M	O	M	M	M	M	-	O	-	M	O	O	M	-	O
R47.2	O	M	O	M	M	O	O	M	O	O	M	M	M	M	-	M	M	O	O	M	O	M	M	M	O	M	M	M	M	M	O	M	M	O	M	M	-	O
AP35a.2	O	M	O	M	M	O	O	M	O	O	M	M	M	M	-	M	M	O	O	M	O	M	M	M	O	M	M	M	M	M	O	M	M	O	M	M	M	O
AP8w.1	O	M	O	M	M	O	O	M	O	M	O	M	M	M	M	M	M	O	O	M	M	M	M	M	O	M	M	M	M	M	O	M	M	O	M	M	-	O
AP5a.3	O	M	O	M	M	O	O	M	O	M	O	M	M	M	M	M	M	O	O	M	M	M	M	M	O	M	O	M	M	O	O	O	M	O	M	M	-	O
Tel VR	O	M	O	M	M	O	O	M	O	M	O	M	M	M	M	M	M	O	O	M	M	M	M	M	O	M	O	M	M	O	M	O	M	O	M	M	M	O

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CHAPTER 2.

Cloning and nucleotide sequence of the catalytic subunit of DNA polymerase-γ of *Neurospora crassa*.

2.1 INTRODUCTION

Though its discovery mitochondrial DNA (mtDNA) four decades ago (Nass *et al.*, 1963; Reich and Luck, 1966), it was recognized as an essential genomic component in eukaryotic cells and consolidated many of the existing basic concepts about cellular and organelle biogenesis, non-Mendelian inheritance, and the molecular basis of mitochondrial diseases (Griffiths, 1992; Shoffner *et al.* 1994; Bourgeron *et al.* 1995). The functional state of mitochondria depends on nuclear-gene encoded factors for mitochondrial gene expression, and mtDNA replication, repair and recombination.

The *Neurospora crassa* mitochondrial genome is approximately 62 kb in size. It encodes 13 mRNAs required for oxidative phosphorylation and two ORFs of unknown function. In addition, it also encodes two ribosomal RNA molecules and 25 tRNAs that are required for translation of mtDNA-encoded mRNAs in the mitochondrial matrix (Mishra, 1991). However, it does not encode any protein involved in the replication of mtDNA.

The replication of mitochondrial DNA appears to be the result of the collaboration of several enzymes, but the basic mechanisms of this event are not fully understood at this time. While replication of the chromosomal DNA is strictly coupled to cell cycle controls, mitochondrial DNA replication is not coupled in the same way. It is regulated to accommodate variations in the rate of mitochondrial biogenesis (Clayton, 1992). The mitochondrial genome is replicated by proteins encoded by nuclear genes, including DNA polymerase-γ (*mip-1*), which is the only polymerase involved in mtDNA replication (Clayton, 1982). DNA polymerase-γ has been cloned from 8 different organisms, including *Saccharomyces cerevisea*, *Schizosacchromyces pombe*, *Pychia pastoris*, *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Drosophila melanogaster*, and *Gallus gallus* (Foury, 1989; Ropp and Copeland, 1995, 1996; Ye *et al.*,1996; Lewis *et al.*, 1996). It is characterized by its resistance to aphidicolin and sensitivity to dideoxynucleotide triphosphates (Bolden *et al.*1977; Knopf *et al.*, 1976; Bertazzoni *et al.*, 1977; Wernette and Kaguni, 1986). It has been shown *in vitro* that purified γ polymerase has 3' → 5' exonuclease activity (Kunkel and Soni, 1988; Kunkel and Mosbaugh, 1989; Insdorf and Bogenhagen, 1989; Olson and Kaguni, 1992).

Here I report the molecular cloning and partial characterization of *mip-1* from *N. crassa*, the gene coding the DNA polymerase-γ of this fungus.

2.2 MATERIALS AND METHODS

2.2.1 Storage of *N. crassa* genomic DNA library.

The Orbach/Sachs cosmid library of *N. crassa* genomic DNA in pMOcosX vector, which has a dominant selectable markers for fungi (hygromycin resistance) and *E. coli* (ampicillin resistance). Cosmid libraries were maintained at –80°C in separate 96-well microtiter plates containing LB medium supplemented with ampicillin.

2.2.2 Design of primers and amplification conditions.

The degenerate primers for amplification of a segment of the DNA polymerase-γ gene by PCR from *N. crassa* genomic DNA where designed on the basis of information derived from highly conserved regions in the corresponding proteins of *Homo sapiens*, *Xenopus laevis*, and *Saccharomyces cerevisiae.* These degenerate primers contain a *Bam*HI restriction site for cloning of the PCR product (Table 2-1). Amplification reactions were initially tested using various concentrations of $MgCl_2$ (2-10 μl of a 25 mM stock), and varying annealing temperatures. Denaturation was for 1 min at 94°C: annealing at 45°C for 1 min; and extension at 72°C for 2 min. This program was repeated through 35 cycles, followed by 5 min at 72°C to ensure completion of the products. Amplified DNA fragments were analyzed by electrophoresis in a 2% agarose (Seakem ME) gel using TAE buffer at 95 volts for 1.5 hr. 5'- and 3'-specific primers (Table 2-1) where subsequently designed on the bases of the nucleotide sequence of the cloned DNA obtained by PCR with degenerate primers from the genomic DNA of *N. crassa*. These two specific primers were used for locating the gene in the cosmid library of *N. crassa* genomic DNA.

2.2.3 Cloning and analysis of PCR products.

Gel-purified, *Bam*HI-digested PCR products were ligated into the plasmid vector pBluescript SK+ (Stratagene) using standard protocols (Sambrook *et. al.*, 1989). Sequencing of DNA was performed by using an ABI Prism DNA sequencer. Analysis of DNA and protein sequences was performed by using the DNASTAR software.

2.2.4 Screening of the *N. crassa* cosmid library.

PCR based screening was performed to identify a single cosmid which contains the DNA polymerase gamma. The Orbach/Sachs pMOcosX cosmid library of *N. crassa* genomic DNA was replica plated in 50 microtiter dishes. Cosmid clones were grown in 200 µl of selection medium (LB-amp, see appendix) at 37°C for about 16 hr and prepared as 50 DNA pools, each containing 96 clones from one of the 50 plates. The pool DNAs were first screened by PCR using primers specific for *N. crass mip-1* DNA (Table 2-1).

Individual positive cosmids were located by PCR and Southern hybridization with row- and column-pool DNAs from the appropriate plates.

Table 2-1 : Sequences of degenerate PCR primers and *N. crassa* specific primers used in this study. Abbreviations represent the following combinations of nucleotides: N=A,C,G, or T; Y=C or T; I=deoxyinosine; K=T or G; H=A or T; M=A or C; D=A,T, or G. The standard one letter code is used for the amino acids. Degenerate primers contain a *Bam*HI site (underlined) for cloning.

Degenerate PCR Primers		
Amino acid Sequence		Primer sequence
(1) GTDLH	5'- primer	GCGGGATCCGGNACNGAYCTNCAY
(2) WTRAMFC	3'- primer	GCGGGATCCRAACATNGCICKNGTCCA
(3) RIYGA	5'- primer	GCGGGATCCCGNATHTAYGGNGCN
(4) HDEIR	3'- primer	GCGGGATCCMCGDATYTCRTCRTG
N. crassa specific primers		
(5) 5'- specific primer		AAGTTCGCATCTCAGCTTCTC
(6) 3'- specific primer		GAAGCGGCGAGTGAGATAGTC

2.2.5 RNA electrophoresis and Northern blotting.

N. crassa mycelium was generated from conidia by 14-hr incubation on a rotary shaker at 25°C, harvested by filtering through Schleicher and Schuell #480 filter paper, and then washed with water and frozen in liquid nitrogen before pulverization. The RNeasy total RNA purification protocol from QIAGEN was used to prepare total RNA from *N. crassa* mycelium. RNA electrophoresis and Northern blotting were performed as described by Sambrook *et al.* (1989). A 3.3-kb *ClaI* fragment of X25:10C which is an internal part of the *mip-1* gene was purified from an agarose gel as described previously (Materials and Methods in Section 1). This gel-purified DNA fragment was used to generate a ^{32}P-labeled probe by using the Random DNA labeling kit from Boehringer Mannheim, Inc.

2.2.6 Restriction Fragment Length Polymorphism (RFLP) mapping.

The location of cosmid DNA on the RFLP map of *N. crassa* was determined as described by Nelson *et al.* (1998). Genomic DNA was prepared from *N. crassa* strains #4450-#4488 (Fungal Genetics Stock Center) and the Mauriceville and Oak Ridge strains according to the method of Lee and Taylor (1990), and 5µg of each DNAs were digested with *Eco*RI. Electrophoresis was carried out to separate the restriction fragments through 0.8% agarose gels before their transfer to a modified nylon membrane for Southern blot analysis. Whole cosmids were used to generate Dig-labeled probes with the kit provided by Boehringer Mannheim, Inc.

2.2.7 DNA sequencing and analysis of the DNA polymerase gamma gene.

DNA sequencing was performed by using an ABI Prism DNA sequencer. Analysis of DNA and protein sequences was performed by using the programs of DNASTAR. The amino acid sequences of the DNA polymerase gamma from different species were aligned using the DNASTAR Megalign program. Some minor adjustments in the alignments were made by visual inspection.

2.3 RESULTS AND DISCUSSION

2.3.1 Cloning of the Catalytic Subunit of *N. crassa* DNA Polymerase-γ

Alignment of mitochondrial DNA polymerases from several species revealed highly conserved regions of amino acids (Figure 2-1). Four of these regions were used for the design of degenerate primers (Table 2-1) for PCR amplification of a portion of the *N. crassa mip-1* gene from genomic DNA. An initial PCR reaction using degenerate primers 1 and 2 on genomic DNA from *N. crassa* resulted in a product of the expected size (~600 bp, data not shown). A second round of PCR was performed using degenerate primers 3 and 4, and it yielded a more obvious product of a lower size (~440 bp, data not shown). This PCR product was isolated from the gel, and ligated into the *Bam*HI site of pBluescript SK+ (Stratagene) and cloned in *E. coli*. One clone, named pGam1, contained an insert of the expected size, and, upon sequencing, was found to encode an amino-acid sequence which showed 63.6% identity to the *S. cerevisiae* DNA polymerase-γ. This fragment was used as a probe to hybridize genomic DNA, and the result confirmed the existence of a corresponding sequence in the *N. crassa* genome (data not shown). When the Orbach/Sachs *N. crassa* genomic library was screened by PCR with specific primers 5 and 6 designed from the sequence of pGam1, plate pools G3, G13, X7, X14, X22, and X25 gave positive results (Figure 2-2). When PCR and hybridizations were performed on row and column pool DNAs from the X25 plate, a single cosmid (X25:10C) was identified as containing the *mip-1* gene (Figure 2-3).

Figure 2-1 Amino acid homology comparison for the DNA polymerase-γ genes of *Homo sapiens* (HS), *Xenopus laevis* (XL), and *Saccharomyces cerevisiae* (SC). Sequences used for primer design are indicated in bold letters and by asterisks. Functionally conservative residues are shown as black letters in a yellow background. The 3'-primer sequence **WTRAMFC** is highly conserved in all three yeast species examined by Ye et. al . (1996).

```
                             [illegible]
Hs GTDLHSKTATTVGISREHAKIFNYGRIYGAGQPFAERLLMQFNHRLTQQEAAEKAQQMYAATKGLRWYRL   997
Xl GTDLHSKTASTVGISREHAKVFNYGRIYGAGQPFAERLLMQFNHRLTQEQAAEKAKQMYAVTKGIRRYIL   967
Sc GTDLHTKTAQILGCSRNEAKIFNYGRIYGAGAKFASQLLKRFNPSLTDEETKKIANKLYENTKG-K----   795
   *****                    *****
   Degenerate 5'primer (1)  Degenerate 5'primer (3)

Hs SDEGEWLVRELN-LPVDRTEGGWISLQDLRKVQRETARKSQWKK-WEVVAERAWKGGTESEMFNKLESIA 1065
Xl SKEGEWLVEELG-ISVERGEENSVNLQDLRKIQKDATKRSRRK--WNLVSRRIWTGGTESQMFNKLETIA 1034
Sc ----------------------------------TKRSKLFK-------KFWYGGSESILFNKLESIA   821

Hs TSDIPRTPVLGCCISRALEPSAVQEE---FMTSRVNWVVQSSAVDYLHLMLVAMKWLFEEFAIDGRFCIS 1132
Xl MSPSPKTPVLGCRISRALEPTAVKGE---FITSRVNWVVQSSAVDYLHLMLVAMKWLFEAYDIDGRFCIS 1101
Sc EQETPKTPVLGCGITYSLMKKNLRANS--FLPSRINWAIQSSGVDYLHLLCCSMEYIIKKYNLEARLCIS   889

    otif C
Hs IHDEVRYLVREEDRYRAALALQITNLLTRCMFAYKLGLNDLPQSVAFFSAVDIDRCLRKEVTMDCKTPSN 1202
Xl IHDEVRYLVHSKDRYRAALALQITNLLTRCMFASRLGIQDVPQSVAFFSAVDIDKCLRKEVTMDCSTPSN 1171
Sc IHDEIRFLVSEKDKYRAAMALQISNIWTRAMFCQQMGINELPQNCAFFSQVDIDSVIRKEVNMDCITPSN   959
    *****                    ******
   Degenerate 3'primer (4)   Degenerate 3'primer (2)
```

Figure 2-2. Screening *Neurospora crassa* genomic library (Orbach/Sachs) by PCR with specific primers 5 and 6 designed from the sequence of pGam1. Plate pools G3, G13, X7, X14, X22, and X25 gave positive results.

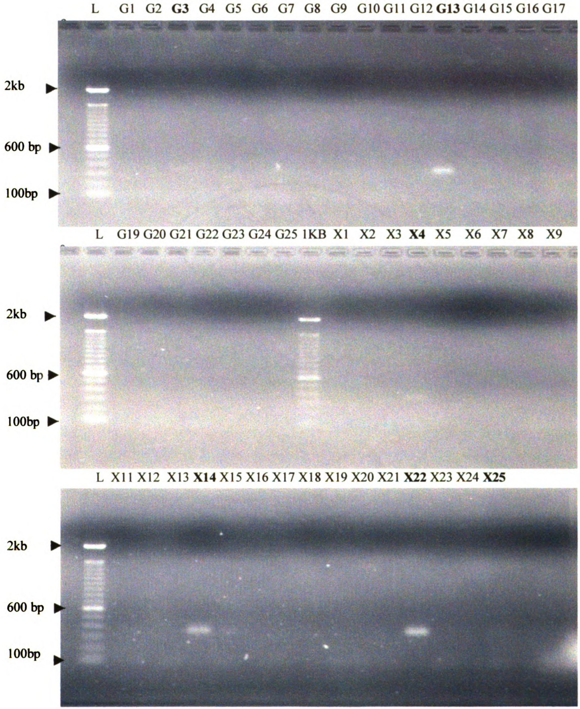
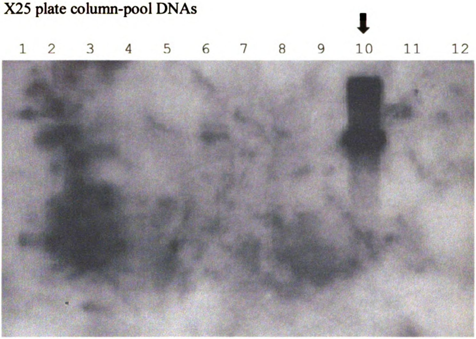
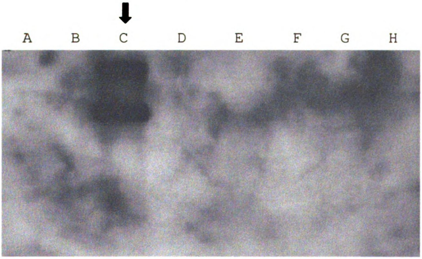


Figure 2-3. Detection of *mip-1*. **(A)** by Southern blot hybridization in column and **(B)** row pools DNAs from cosmid DNA plate X25. The 350-bp *mip-1* specific probe detected the column 10 and row C pool DNAs. **(C)** PCR screening of plate X25 row- and column-pool DNAs was done with specific primers 5 and 6. It also detected row C and column 10 in the X25 plate.

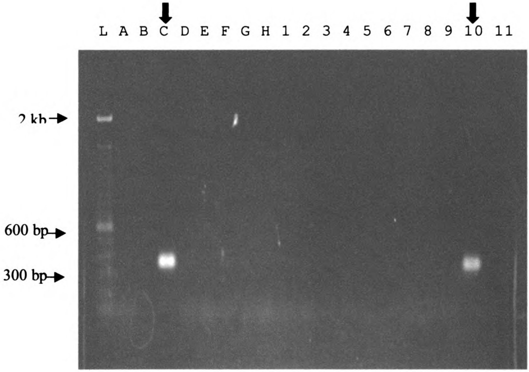
(A) X25 plate column-pool DNAs



(B) X25 plate row-pool DNAs



(C) PCR detection of *mip-1* from row- and column-pool DNAs of plate X25.



2.3.2 Nucleotide Sequence of *mip-1*

The nucleotide sequence of the *mip-1* gene was determined by directly sequencing cosmid X25:10C outwards with a series of synthetic primers from the segment corresponding to the DNA cloned in pGam1. The sequence of *mip-1* is deposited in GenBank under accession number AF111068. The gene has a 3918-nucleotide open reading frame (ORF) encoding a protein of 1305 amino acids (146 kDa), and contains no introns. A mitochondrial targeting sequence and a cleavage site for a mitochondrial presequence were found in the N-terminal region of putative protein (Figure 2-4).

The DNA polymerase-γ from *Neurospora* has a high degree of homology with the other eight polymerases of its type that have been characterized so far (Table 2-2 and Figure 2-5). These proteins all contain the three exonuclease motifs (Figure 2-4 and 2-5) identifying an editing domain and the three motifs characteristic of the family-A DNA polymerases. Ropp and Copeland (1996) reported five sequence insertion sites in higher eukaryotes. However, the alignment shown in this paper (Figure 2-6) demonstrates that, relative the fungal proteins, higher eukaryotic gamma DNA polymerases have 8 insertion sites (inserts A-H) totaling approximately 200 amino-acid residues. Insertion sites A and B are located between the Exo I and Exo II domains, inserts C, D, E, and F are located between the last exonuclease domain and the first polymerase domain, one insertion site, G, is situated between polymerase motifs B and C, and the last insert, H, is located after polymerase motif C near the N-terminus of the insect and vertebrate polymerases. In addition, the *N. crassa* and *S. cerevisiae* polypeptides have C-terminal extensions of approximately 200-270 amino-acids relative to all the other gamma DNA polymerases. Nonetheless, the similarity between the amino-acids sequences in the extensions of the yeast and *Neurospora* protein is relatively low when compared to the similarity in the regions spanning the polymerase and exonuclease domains. The significance, if any, of this extension is unclear. However, two potential cAMP- and cGMP-depentent protein kinase phosphorylation sites, KKTT(1248-1251) and KKET(1259-1262), are found in the extension of the *N. crassa* DNA polymerase-γ. The *S. cerevisiae* polymerase-γ polypeptide also has four potential cAMP- and cGMP-dependent protein kinase phosphorylation sites in the C-terminal extension, which are KKLT(1137-1140), KKNT(1142-1145), KKPS(1204-1207), and RKSS(1232-1235). All the γ-DNA polymerases share potential phosphorylation and glycosylation sites in the middle of proteins (Figure 2-6), but it is not clear whether or not the proteins are phosphorylated or glycosylated *in vivo*.

Figure 2-4. Nucleotide and amino acids sequences of the *N. crassa mip-1* gene. Putative regulatory elements including CAAT box (red), TATA box (blue), *N. crassa* transcription consensus starting point (TCATCANC; Bruchez et al. 1993) are bold and indicated with asterisks below the sequence. The cleavage site for the prepeptide is highlighted in yellow in the amino acid sequence and the cleavage point is indicated by an arrow. The locations of the degenerate primers are highlighted in pink in the amino acid sequence and two *mip-1* specific primers are highlighted in green in the nucleotide sequence.

```
gtcataacgccatgatgcttttcaacgccgtagtgactacgatctggactggatcgtcaaggacagtgtcataatgacact   81

tctatctgagatcattagttggttaccaacctaagcgccatgagtatgttcatgtttttcgtcagccaggtgctgcgtat  162

tccattttcgtttagtgggtagcccgctatctgacccttgaatggtgagagattcgcgcaagtgagtctagcccaacaaga  243

ctgattgaggaactcaaacccaaagtaacagaagcaatcgaagctcaactggacaggatctcaagtgaaaatagagacaag  324

atacaatgcccacgtgtgagtgtggtgacgagctgatcaaagagtgagagggaggtagtacttgggtacatgggtacaagc  405

tagtagcgtgcctctaactgaggggccgggatcccaattggcatggactggaacttttctgaacttttttttttttttt  486

tttttttttttttttttttttttgttggacctgcgaatgcccatgacatcgatgatggattcggtacggtcgccgccccg  567

caatcgcactcaaaccaccacttcgatatccaccacaattcattcagtctctataaccagtcaccgtggcgtatcaccact  648
****                                               *******              *******
ttccagactgaaaacacgcagcctaccatgacttgaaatccgacttccagctggtgattatctagaatgctgacccccgtc  729
                                                                  M  L  T  P  V      5

cgctgccggacggtaccaaatgcgactgttgcgaccgccgcccgagtccttcgccgagccaacctcttttcccgatatcct  810
R  C  R  T  V  P  N  A  T  V  A  T  A  A  R  V  L  R  R  A  N  L  F  S  R  Y  P     32

cgacagctcggacacctacgatgggactcgactatcgcccaagtgctcgagcgcaagggcctcggcgtcccctccacagcg  891
R  Q  L  G  H  L  R  W  D  S  T  I  A  Q  V  L  E  R  K  G  L  G  V  P  S  T  A     59

cgccataacgagatcggtgtccagcagctcagcgagcacctctacaagcagcttttcccccgcggaaacaccgaccctcct  972
R  H  N  E  I  G  V  Q  Q  L  S  E  H  L  Y  K  Q  L  F  P  R  G  N  T  D  P  P     86
cccgaactcgctatcgagctggccaaggaccaccttgcccgccatgacttgctgggaaagaccaccgataagactcctcct 1053
A  P  E  L  I  E  L  A  K  D  H  L  A  R  H  D  L  L  G  K  T  T  D  K  T  P  P    113

atcgcatttcaactaccggccttggtgggcgataccctcgacgaacacttccacaagctgggcgtcgacgccgccgaaccc 1134
I  A  F  Q  L  P  A  L  V  G  D  T  L  D  E  H  F  H  K  L  G  V  D  A  A  E  P    140

ttccttacccatgccaagcagttcgcagacgcccatctacccccaagccgacgagttgggtacggaggagtggctggaca 1215
F  L  T  H  A  K  Q  F  A  D  A  H  L  P  P  K  P  T  S  W  V  R  R  S  G  W  T    167

aaatacaaccgcgatggcacgaccgagaacgacgtcctgccccagggcaacatgatgtgcttcgatgtggaggtcatgtat 1296
K  Y  N  R  D  G  T  T  E  N  D  V  L  P  Q  G  N  M  M  C  F  D  V  E  V  M  Y    194

aaggataacccctatgctgtcatggcatgtgctggtactccggatgcctggtatgcctggctgtcgccgtggctgcttgga 1377
K  D  N  P  Y  A  V  M  A  C  A  G  T  P  D  A  W  Y  A  W  L  S  P  W  L  L  G    221

gagacggaaaataaggcccagttggtaccaatgggcgatccaaccgtcgacagaatcatcgtgggtcacaacattggctac 1458
E  T  E  N  K  A  Q  L  V  P  M  G  D  P  T  V  D  R  I  I  V  G  H  N  I  G  Y    248

gaccgtgccaagatactggaggaatacgacctgaagcagacgaggaacttctttcttgacaccatgtctctgcatgtcgct 1539
D  R  A  K  I  L  E  E  Y  D  L  K  Q  T  R  N  F  F  L  D  T  M  S  L  H  V  A    275

gtgaacggaatgtgctcccaacaacgaccgacgtggatgaaacacaagaaagccagagaactacgggagaaggcagagcat 1620
V  N  G  M  C  S  Q  Q  R  P  T  W  M  K  H  K  K  A  R  E  L  R  E  K  A  E  H    302

gaaagtgccagtgtagaactgcaagaagtcttgcagggcggttccctcacggccgaggaggccgacctctgggtcgataaa 1701
E  S  A  S  V  E  L  Q  E  V  L  Q  G  G  S  L  T  A  E  E  A  D  L  W  V  D  K    329

agctccatcaactcgctaagagacgtggcccaattccacctcaacgtcaagatcgacaaggatatcagggatgtctttgcg 1782
S  S  I  N  S  L  R  D  V  A  Q  F  H  L  N  V  K  I  D  K  D  I  R  D  V  F  A    356

gaaacagatcgaaatgtgattctgaaccagctggacgatctcctgacttactgtgcggcagatgttcaagtcacccaccag 1863
D  R  E  T  N  V  I  L  N  Q  L  D  D  L  L  T  Y  C  A  A  D  V  Q  V  T  H  Q    383

gtttaccaggtcgtgttccctaactttctcggagtctgtcctcatcccgtcagctttgccgctctacgccacctcgcctcc 1944
V  Y  Q  V  V  F  P  N  F  L  G  V  C  P  H  P  V  S  F  A  A  L  R  H  L  A  S    410

gtcattctgcccgtcaacaagacttgggatacttatatcgagacagcggaggcaacatacctgcaaatgttacacggcgtt 2025
V  I  L  P  V  N  K  T  W  D  T  Y  I  E  T  A  E  A  T  Y  L  Q  M  L  H  G  V    437

caggagcgccttttcactctgatggagaggacgctggattataaggccgaccctgaaaaatatctttctgatccttggttg 2106
Q  E  R  L  F  T  L  M  E  R  T  L  D  Y  K  A  D  P  E  K  Y  L  S  D  P  W  L    464

agccaactagactggtcaggacaggagatcaagatggccaagcccaagaaaaagggcgacgtggaacggccagccctgaat 2187
S  Q  L  D  W  S  G  Q  E  I  K  M  A  K  P  K  K  K  G  D  V  E  R  P  A  L  N    491
```

cagaaactaccaggctatccccaatggtacaaggatctgtttgtaaaggttcccaaggagctctcgggcttggacgaaccc 2268
Q K L P G Y P Q W Y K D L F V K V P K E L S G L D E P 518

gacaaagagcaggaaaatagaaaggctcgacatgaattcatcaatctcaccgtccgttcacggatagccccgttgctcctc 2349
D K E Q E N R K A R H E F I N L T V R S R I A P L L L 545

aaattatcatgggaaggctaccccttattctggtccgatcaatttggctggacgttccaggtcccacgagaaaaagcagag 2430
K L S W E G Y P L F W S D Q F G W T F Q V P R E K A E 572

acctttattcagcgccagatgacacctgttcaatttgaggacccagatgtcgatgatcgacttaggatggatgttgatcac 2511
T F I Q R Q M T P V Q F E D P D V D D R L R M D V D H 599

aagtacttcaaacttcctcacaaagatggcccgaacgctagatgtgtcaacccaatggccaagggttatcttccctacttt 2592
K Y F K L P H K D G P N A R C V N P M A K G Y L P Y F 626

gaaaagggcatcctttcttccgagtacccctacgcaaaggaagccctggaaatgaatgcttcttgttcatactggatcagt 2673
E K G I L S S E Y P Y A K E A L E M N A S C S Y W I S 653

gctcgagagagaatcaagaatcagatggttgtctatgaagatcagcttcctccgtctcagagatttgtcaacaaggatgca 2754
A R E R I K N Q M V V Y E D Q L P P S Q R F V N K D A 680

gacagcaacacccctattggcggctttgttcttccccaggtcattcctatgggtaccattactcgtcgtgctgtcgagaga 2835
D S N T P I G G F V L P Q V I P M G T I T R R A V E R 707
acatggttgacggcatccaatgccaagaagaaccgtgttggatcagagctcaaagccatggttcgcgcaccaccaggttat 2916
T W L T A S N A K K N R V G S E L K A M V R A P P G Y 734

gtctttgtcggtgccgatgttgactcggaagaactttggattgcctctgttgttggagacgctactttcaaactgcatggt 2997
V F V G A D V D S E E L W I A S V V G D A T F K L H G 761

ggtaatgccatcggcttcatgacccttgagggtaccaaatcccagggcactgatctccacagtcggacggcttccattctg 3078
G N A I G F M T L E G T K S Q G [illegible] H S R T A S I L 788
→Degenerate 5′primer (1)

ggcatcacccgtaatgacgcaaaagtgtttaactatggccgtatctacggcgccggtctc[redacted] 3159
G I T R N D A K V F N Y G R [illegible] A G L K F A S Q L L 815
→ Degenerate 5′primer(3) → 5′specific primer (5)

cggcagttcaaccctagcttgaccgaggcggaaacgactgccatcgcgacaaagctctacgatgccaccaaaggtgccaaa 3240
R Q F N P S L T E A E T T A I A T K L Y D A T K G A K 842

actaaccgcaagagcctttacaagcggcccttctggcgaggcggtaccgagtccttcgtgtttaacatgcttgaagagttt 3321
T N R K S L Y K R P F W R G G T E S F V F N M L E E F 869

gccgagcaagagcgtcctcgtaccccggttctcggtgcgggcatcacagaggccctcatgagccgctgggtcagcaaaggc 3402
A E Q E R P R T P V L G A G I T E A L M S R W V S K G 896

gggttcctgacttcgcgtatcaactgggccatccaatctagtggtgtcgattaccttcacttgctcatcatcgctatg[redacted] 3483
G F L T S R I N W A I Q S S G V D Y L H L L I I A M D 923

[redacted]aacctcgcctgccggttggccatcactgtgcacgacgaaatccgctatcttgccgaggaacct 3564
Y L T R R F N L A C R L A I T V H [illegible] R Y L A E E P 950
← 3′ specific primer (6) ← Degenerate 3′primer (4)

gacaaataccgggtagccatggcactgcagattgccaacctgtggactcgtgtcatgttcgcgcagcaagtcggcattcaa 3645
D K Y R V A M A L Q I A N L W [illegible] A Q Q V G I Q 977
←Degenerate 3′primer (2)

gatctaccgcagtcctgcgccttcttcagcgctgtggacatcgaccacgtgcttcggaaggaggtcgacatggattgtatc 3726
D L P Q S C A F F S A V D I D H V L R K E V D M D C I 1004

acgcctagtaacccgatacccattgcgcacggcgagagcatcgatatcttccagatcctggagaagggagacgacgcaaag 3807
T P S N P I P I A H G E S I D I F Q I L E K G D D A K 1031

ctggatgacagcattgtccctcagtctcaatatgcacctcgcctggagaacatcccgtatacgcctcgtgtgcctgtgatg 3888
L D D S I V P Q S Q Y A P R L E N I P Y T P R V P V M 1058

cagaggcttcgcgagagggccgaggccggcgatcaccaagccttccttcggttcatccgggcacagattaccaattccgat 3969
Q R L R E R A E A G D H Q A F L R F I R A Q I T N S D 1085

```
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E  E  L  K  R  I  I  A  E  T  R  Y  S  D  P  Y  G  A  F  S  L  A  S  N  G  R  V   1112

tcaggcaatccacatcagcggcatgccgctgtacatgcttcgacaaagacggctgcggctccttcaaaccttccatcgca 4131
S  G  N  P  H  Q  R  H  A  A  V  H  A  S  T  K  T  A  A  A  P  S  K  P  S  I  A   1139

agtcgtttcgattccgtctcgcaggcatcgaggatcaagtccgtcgcagctggcagtgatgagcccaccatcagagcgacc 4212
S  R  F  D  S  V  S  Q  A  S  R  I  K  S  V  A  A  G  S  D  E  P  T  I  R  A  T   1166

aaagcgcagggcaaagccatggcaaaagccagtggtacaaaacttgctgcctccacgaaggataccgtcctcaacgtaacg 4293
K  A  Q  G  K  A  M  A  K  A  S  G  T  K  L  A  A  S  T  K  D  T  V  L  N  V  T   1193

atcaagaagaaggtggcggcccccgagatggcggctgttccgtcaacctcttctgaatctaagtccaaggcttcagccaca 4374
I  K  K  K  V  A  A  P  E  M  A  A  V  P  S  T  S  S  E  S  K  S  K  A  S  A  T   1220

acaagcacgacaaccaccgagaacgccaccgcatccccttcatcctcatcgaacgtcgatgcaaagaaaaccacatccaaa 4455
T  S  T  T  T  T  E  N  A  T  A  S  P  S  S  S  S  N  V  D  A  K  K  T  T  S  K   1247

accaagccgacccacaagaaggagactgaaggggaaccatttccctcccttgacgaccccgtcatcgccgcccgcctcgaa 4536
T  K  P  T  H  K  K  E  T  E  G  E  P  F  P  S  L  D  D  P  V  I  A  A  R  L  E   1274

gccgtctccaagacttcaccagggaccagggcttccgtcgccgcgaagctagacgcccttgcctccttttgccatgcgagc 4617
A  V  S  K  T  S  P  G  T  R  A  S  V  A  A  K  L  D  A  L  A  S  F  C  H  A  S   1301

tgctgcggctgctgaggctgctgtcaccaccaccactactccagagcacgccaaccaaccctcccccgttgctcccaaggc 4698
C  C  G  C  ***                                                                   1305

aaaggaacccacaaccaccaactggcagaaaaggtctactgaaacctaccgccgtccccaaaaaccccactcctactctca 4779

cgcccactcaccaaaaagtctaatccacattcacaccaaccacaccaaaacccgtcggtcgtcctcgtacactcccccatc 4860

ctcggctacaccgctcccaagaaaaag                                                       4887
```

Table 2-2. Percent similarity among 9 different γ-DNA polymerases. Nc, *Neurospora crassa* ; Pp, *Pichia pastoris*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*.

Nc	Pp	Sc	Sp	Hs	Mm	Xl	Dm	Gg	
***	41.8	37.3	45.6	29.2	29.5	29.6	26.8	36.0	Nc
	***	48.4	41.9	30.8	29.9	30.6	26.5	31.2	Pp
		***	41.9	23.9	24.2	22.5	21.7	33.4	Sc
			***	31.7	31.6	32.1	27.4	32.0	Sp
				***	82.4	64.8	39.3	74.7	Hs
					***	63.5	37.4	74.0	Mm
						***	37.0	70.8	Xl
							***	39.3	Dm
								***	Gg

Figure 2-5. Linear map of the *N. crassa mip-1* gene. The ORF is represented by a wide grey bar. Putative regulatory elements including CAAT box, TATA box, *N. crassa* transcription consensus starting point (TCATCANC; Bruchez *et al.* 1993), and the possible cleavage site for the mitochondrial presequence are indicated. Conserved exonuclease and polymerase motifs are shown as labelled black boxes.

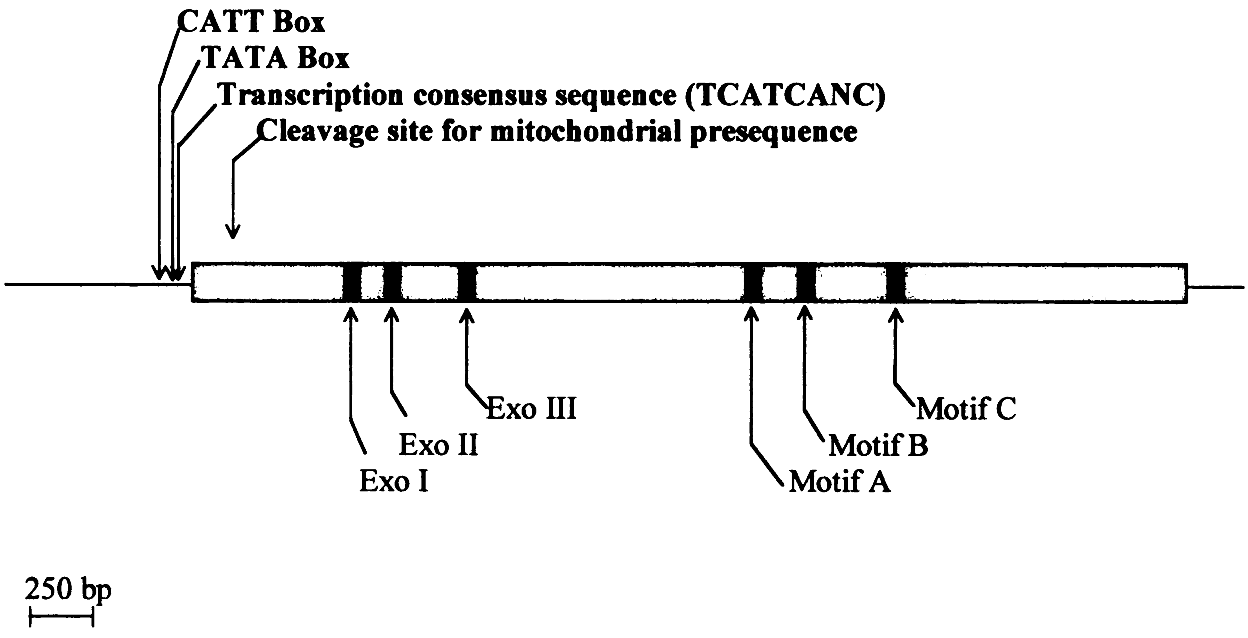
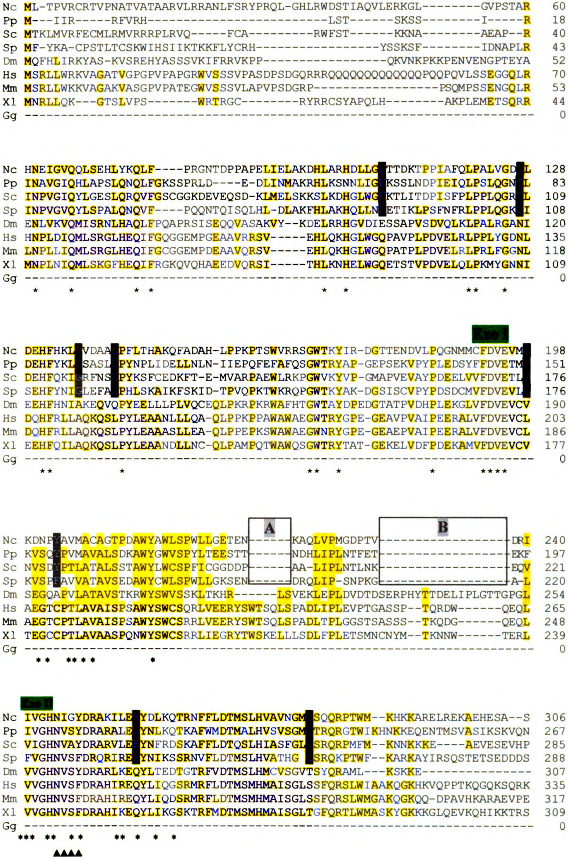


Figure 2-6. Multiple alignment of DNA polymerse-γ sequences. Amino acid-sequences were aligned with the DNASTAR program. Functionally conservative residues are shown as black letters in a yellow background and the residues that are identical in all the polymerases are marked with asterisks below the sequences. Amino acids that are highly conserved, but different in the fungal polymerases from those that appear at the corresponding position in the animal polymerases, are shown in white letters in a black background. The locations of exonuclease and polymerase domain sequences are indicated above the aligned sequences. The mammalian and insect DNA polymerases have 8 insertion sites (inserts A-H) relative to the fungal polymerases. The amino acid sequences that were used to generate of four degenerate primers are indicated by arrows below the aligned sequences The possible glycosylation site is indicated by black triangles (▲). A possible Y-phosphorylation site is indicated by black dot (●). Nc, *Neurospora crassa* (AF111068); Pp, *Pichia pastoris* (U49510); Sc, *Saccharomyces cerevisiae* (J05117); Sp, *Schizosaccharomyces pombe* (Z47976); Hs, *Homo sapiens* (U60325); Mm, *Mus musculus* (U53584); Xl, *Xenopus laevis* (U49509); Dm, *Drosophila melanogaster* (U60298); Gg, *Gallus gallus* (U60297).



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Nc  ML-TPVRCRTVPNATVATAARVLRRANLFSRYPRQL-GHLRWDSTIAQVLERKGL--------GVPSTAR  60
Pp  M---IIR-----RFVRH-------------------------LST------SKSS--------I-----R  18
Sc  MTKLMVRFECMLRMVRRRPLRVQ-----FCA---------RWFST------KKNA--------AEAP--R  40
Sp  MF-YKA-CPSTLTCSKWIHSIIKTKKFLYCRH-----------------YSSKSF--------IDNAPLR  43
Dm  MQFHLIRKYAS-KVSREHYASSSVKIFRRVKPP-----------------QKVNKPKKPENVENGPTEYA  52
Hs  MSRLLWRKVAGATVGPGPVPAPGRWVSSSVPASDPSDGQRRRQQQQQQQQQQQQQPQQPQVLSSEGGQLR  70
Mm  MSRLLWKKVAGAKVASGPVPATEGWVSSSVLAPVPSDGRP-----------------PSQMPSSENGQLR  53
Xl  MNRLLQK---GTSLVPS-------WRTRGC--------RYRRCSYAPQLH--------AKPLEMETSQRR  44
Gg  ----------------------------------------------------------------------   0


Nc  HNEIGVQQLSEHLYKQLF----PRGNTDPPAPELIELAKDHLARHDLLG TTDKTPPIAFQLPALVGD L 128
Pp  INAVGIQHLAPSLQNQLFGKSSPRLD----E-DLINMAKRHLKSNNLIG KSSLNDPIEIQLPSLQGN L  83
Sc  INPVGIQYLGESLQRQVFGSCGGKDEVEQSD-KLMELSKKSLKDHGLWG KTLITDPISFPLPPLQGR L 109
Sp  INPVGVQYLSPALQNQVF----PQQNTQISQLHL-DLAKFHLAKHQLLN ETIKLPSFNFRLPPLQGK I 108
Dm  ENLVKVQMISRNLHAQLFPQAPRSISEQQVASAKVY--KDELRRHGVDIESSAPVSDVQLKLPALRGANI 120
Hs  HNPLDIQMLSRGLHEQIFGQGGEMPGEAAVRRSV-----EHLQKHGLWGQPAVPLPDVELRLPPLYGDNL 135
Mm  LNPLLIQMLSRGLHEQIFGCGGEMPDEAAVQRSV-----EHLQKHGLWGQPATPLPDVELRLPRLFGGNL 118
Xl  MNPLNIQMLSKGFHEQIFRGKQVQHAEEDVQRSI-----THLKNHELWGQETSTVPDVELQLPKMYGNNI 109
Gg  ----------------------------------------------------------------------   0
     *    *        * *                       *  *                **   *


                                                                 Exo I
Nc  DEHFHKL VDAA PFLTHAKQFADAH-LPPKPTSWVRRSGWTKYIR-DGTTENDVLPQGNMMCFDVEVM  198
Pp  DEHFYKL SASL PYNPLIDELLNLN-IIEPQFEFAFQSGWTRYAP-GEPSEKVPYPLEDSYFFDVETM  151
Sc  DEHFQKIGRFNS PYKSFCEDKFT-E-MVARPAEWLRKPGWVKYVP-GMAPVEVAYPDEELVVFDVETL  176
Sp  SEHFYNIGLEFA PHLSKAIKFSKID-TPVQPKTWKRQPGWTKYAK-DGSISCVPYPDSDCMVFDVEVL  176
Dm  EEHFHNIAKEQVQPYEELLLPLVQCEQLPKRPKRWAFHTGWTAYDPEDGTATPVDHPLEKGLVFDVEVCV 190
Hs  DQHFRLLAQKQSLPYLEAANLLLQA-QLPPKPPAWAWAEGWTRYGPE-GEAVPVAIPEERALVFDVEVCL 203
Mm  DQHFRLLAQKQSLPYLEAAASLLEA-QLPPEPKSWAWAEGWNRYGPE-GEAEPVAIPEERALVFDVEVCL 186
Xl  EEHFQILAQKQSLPYLEAANDLLNC-QLPAMPQTWAWQSGWTRYTAT-GEKELVDFPDEKAMVFDVEVCV 177
Gg  ----------------------------------------------------------------------   0
      **          *                         ** *            *      ****


                                     A                    B
Nc  KDNPYAVMACAGTPDAWYAWLSPWLLGETEN-----KAQLVPMGDPTV-------------------DRI 240
Pp  KVSQYPVMAVALSDKAWYGWVSPYLTEESTT-----NDHLIPLNTFET-------------------EKF 197
Sc  NVSDYPTLATALSSTAWYLWCSPFICGGDDP-----AA-LIPLNTLNK-------------------EQV 221
Sp  KVSPFAVVATAVSEDAWYCWLSPWLLGKSEN-----DRQLIP-SNPKG-------------------A-L 220
Dm  SEGQAPVLATAVSTKRWYSWVSSKLTKHR------LSVEKLEPLDVDTDSERPHYTTDELIPLGTTGPGL 254
Hs  AEGTCPTLAVAISPSAWYSWCSQRLVEERYSWTSQLSPADLIPLEVPTGASSP--TQRDW------QEQL 265
Mm  AEGTCPTLAVAISPSAWYSWCSRRLVEERYSWTSQLSPADLTPLGGSTSASSS--TKQDG------QEQL 248
Xl  TEGCCPTLAVAASPQNWYSWCSRRLIEGRYTWSKELLLSDLFPLETSMNCNYM--TKNNW------TERL 239
Gg  ----------------------------------------------------------------------   0
     **   ** * *       *


   Exo II
Nc  IVGHNIGYDRAKILE YDLKQTRNFFLDTMSLHVAVNGM SQQRPTWM--KHKKARELREKAEHESA--S 306
Pp  IVGHNVSYDRARALE YNLKQTKAFWMDTMALHVSVSGM TRQRGTWIKHNKKEQENTMSVASIKSKVQN 267
Sc  VIGHNVAYDRARVLE YNFRDSKAFFLDTQSLHIASFGL SRQRPMFM--KNNKKKE----AEVESEVHP 285
Sp  FVGHNVSFDRQRIRE YNIKSSRNVFLDTMSLHVATHG  SRQKPTWF--KARKAYIRSQSTETSEDDDS 288
Dm  VVGHNVSYDRARLKEQYLTEDTGTRFVDTMSLHMCVSGVTSYQRAML---KSKKE--------------- 307
Hs  VVGHNVSFDRAHIREQYLIQGSRMRFLDTMSMHMAISGLSSFQRSLWIAAKQGKHKVQPPTKQGQKSQRK 335
Mm  VVGHNVSFDRAHIREQYLIQDSRMRFLDTMSMHMAISGLSSFQRSLWMGAKQGKQQ-DPAVHKARAEVPE 317
Xl  VVGHNVSFDRAHIKEQYLIKGSKTRFMDTMSMHMAISGLTGFQRTLWMASKYGKKKGLQEVKQHIKKTRS 309
Gg  ----------------------------------------------------------------------   0
   *** ** **    ** * * *
        ▲▲▲▲
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```
Nc VELQEVLQGGSLTAE--EADLWVDKSSINSLRDVAQ█HLN-█KIDKDIRDVF█ET█RNVILNQLDDLLTY  373
Pp EELQELLDSTSIEDPMLEDNPWINKSSLNSLERVAE█YCK-█KLKKDIRNSF█TE█PDELRGNFDELMQY  336
Sc E--------ISIED---YDDPWLNVSALNSLKDVAK█HCK-█DLDKTDRDFF█ST█KSTIIENFQKLVNY  343
Sp SSFDDDYQN-YLKQE--P---WLAHSSVNSLKDVAK█HCN-█TLDKSKRDDF█SL█KEPILQKLNELITY  351
Dm ----PAAEDLG----------WLEQSSLNSLVELHRLYCGGDTLSKEPRNIFVEGTLEQVRQSFQSLTNY  362
Hs ARRGPAISSWD----------WLDISSVNSLAEVHRLYVGGPPLEKEPRELFVKGTMKDIRENFQDLMQY  395
Mm ESQWSSDSSWD----------WMDISSANNLADVHNLYVGGPPLEKEPRELFVKGSMRDIRENFQDLMQY  377
Xl NFSGSPISSWD----------WVNISSINNLADVHALYVGGPPLEKEARELFVKGSMSDIRTEFQELMRY  369
Gg ----------------------------------------------------------------------    0
                         *   *  * *            * *     *              *  *

Nc CAADVQVTHQVYQVVFPNFLGVCPHPVSFAALRH█ASVILPVN-KTWDTYIETA█ATYLQMLHGVQERLF  442
Pp CAKDVFATGKVFQKVYPKFKKLIPHPVTLAALKD█SSCILPTT-TKWEDYIETS█RLYQESRRMIEKNLH  405
Sc CATDVTATSQVFDKIFPVFLKKCPHPVSFAGLKS█SKCILPTKLNDWNDYLNSS█SLYQQSKVQIESKIV  413
Sp CAHDTYSTHQVFKKVFPQFLEVCPHPATFSAMLS█GSVFLPVN-HSWTRYINGV█EQYQQMIQLVDQKLS  420
Dm CASDVEATHRILRVLYPLYAERFPHPASLAGMLEMGSAYLPVN-SNWERYIREAQLTYEDLSIEAKYHLG  431
Hs CAQDVWATHEVFQQQLPLFLERCPHPVTLAGMLEMGVSYLPVN-QNWERYLAEAQGTYEELQREMKKSLM  464
Mm CARDVWATFEVFQQQLPLFLERCPHPVTLAGMLEMGVSYLPVN-QNWERYLTEAQNTYEELQRDDEEVVV  446
Xl CALDVQATHEVFQEQFPLFMERCPHPVTLSGMLEMGVSYLPVN-QNWERYLDEAQTSYEELQKEMKKSLM  438
Gg ----------------------------------------------------------------------    0
   ** *            *      ***             **     *  *       *

                                                          C
Nc TLMERTLDYKADPEK-YLSDPWLS█LDWSGQEIKMAKPKK------------------------------  482
Pp VICEETVKLKDDPTKPWENDPWLS█LDWTIDPIRLTK---------------------------------  443
Sc QIIKDIVLLKDKPDF-YLKDPWLS█LDWTTKPLRLTK---------------------------------  450
Sp QYAEKAKDLINTKDT-VLKDPWLR█LDWT--PCNLYRKLK------------------------------  458
Dm RRAEEACSLLLDD--QYRQNLWLWDEDWSVQELKLKQPPKRK-------------------PLPTVELKD  480
Hs DLANDACQLLSGE--RYKEDPWLWDLEWDLQEFKQKKAKKVKKEPATASKLPIEGAGAPGDPM---DQED  529
Mm VWLMMPGQLLSGE--RYKEDPWLWDLEWDLQEFKQKKAKKVKK-PASASKLPIEGAGPFGDPM---DQED  510
Xl KLANDACQLLTKD--AYKEDPWLWDLEWDIQESKQKKTKISKKQ-----KKANEAAESVGNKLVEDHNED  501
Gg ----------------------------------------------------------------------    0
                         **    *

                 C
Nc ------KGDVER------------------PALN-QKLPGYPQWYKDLFVKVP-KELSGLDEPDKEQENR  525
Pp ------KGEI--------------------HKN-QKLPGYPNWYKQLIVK---NEL-------------  469
Sc ------KGV---------------------PAKC-QKLPGFPEWYRQLFPS---KDTVEP----------  479
Sp -------------------------------KAT-QEVPVVPKWYKKAYCKTE-KRAV------------  483
Dm SGNTPEERRLQ-AKFQHLYDQQ------ALLPARRPLLPGYPLWYRKLCRKPPAKRADEILE--DDEEPW  541
Hs LGPCSEEEEFQQDVMARACLQKLKGTT-ELLPKRPQHLPGHPGWYRKLCPRL-------------DDPAW  585
Mm PGPPSEEEELQRSVTAHNRLQQLRSTT-DLLPKRPQHLPGHPGWYRKLCPRL-------------DDPAW  566
Xl PGPPTEKEE-SRPSMGKLYLEDLKLKTLPLLPKRNQHLPGHPGWYRKLCPKL-------------EDPDW  557
Gg ------------------------------------------------------------------EEGW    4
                                         *  * **

                                                              D
Nc KARHEFINL█VR█RIAPLLLKLSWEGYPLF█SDQFGWTFQVPR---E-----------------------  570
Pp -------KL█TK█RTSPLLLKLAWNGNPLY█IQTQGWCFKVPKNKTE-----------------------  509
Sc -------KI█IK█RIIPILFKLSWENSPVI█SKESGWCFNVPHEQVE-----------------------  519
Sp --------I█AK█RLAPILLRLKWKKHPLA█SDTYGWVFSVERTSKD-----------------------  522
Dm SPGASEISTGM--QIAPKLLSLCWEGYPLHYEREQGWGFLVPFRSDSEGVDRL--PMEQLLAHCPVPEFA  607
Hs TPGPSLLSLQM--RVTPKLMALTWDGFPLHYSERHGWGYLVPGRRDNLAKLPTGTTLESAGVVCPYRAIE  653
Mm APGPSLLSLQM--RVTPKLMALTWDGFPLHYSDSHGWGYLVPGRRDNLTEPPVSPTVESAAVTCPYRAIE  634
Xl LPGPGLISLQM--RLTPKLMRLTWDGYPLHYSEKHGWGYLVPGRKNN----KLNNEEEEEIIPCPYRAIE  621
Gg VPGPSLISLQM--RVTPKLMRLAWDGFPLHYSEKHGWGYLVPGRQDNLP----AASAEPEGPVCPHRAIE   68
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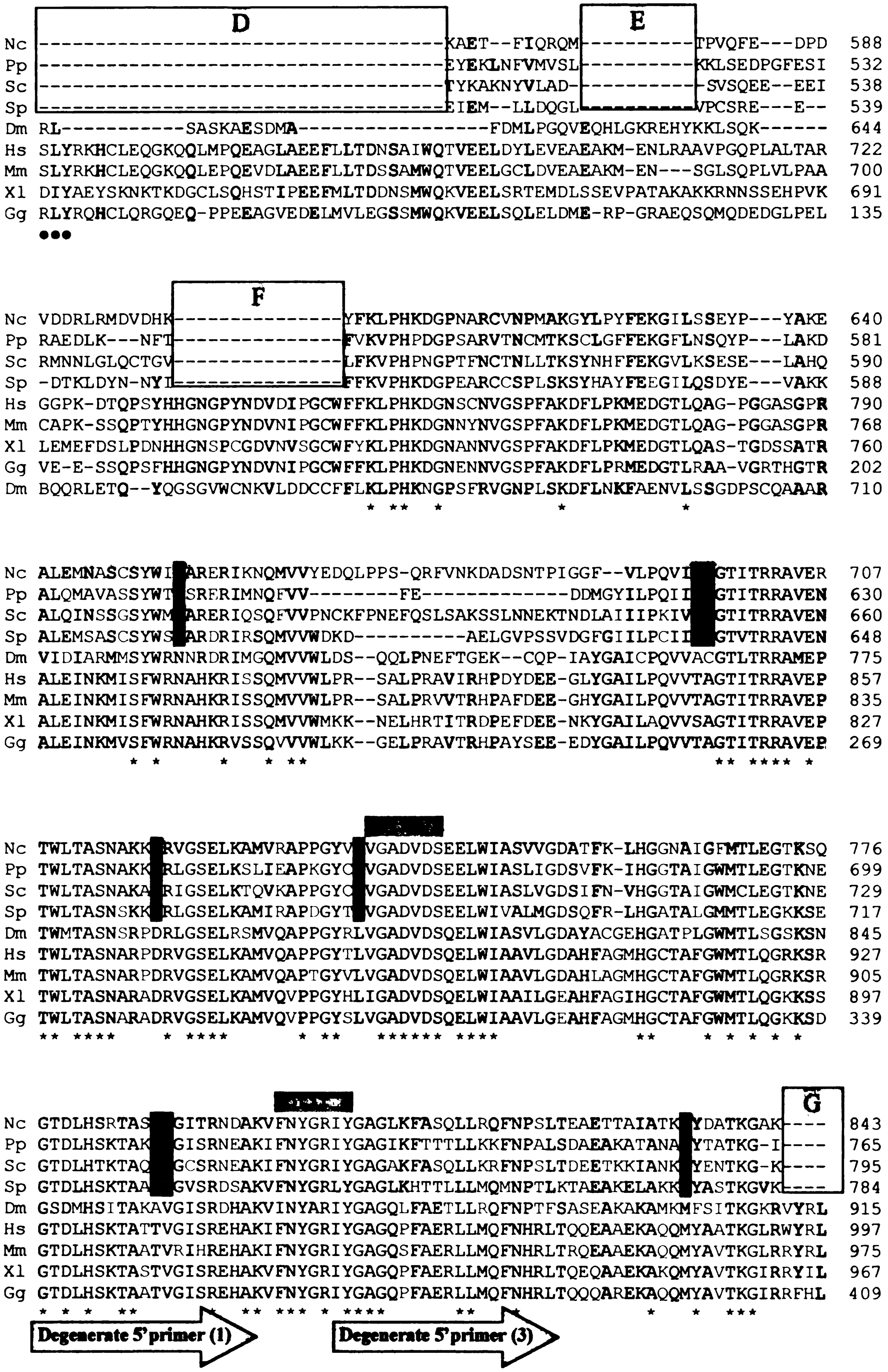

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Pp ------------------------------------EYEKLNFVMVSL----------KKLSEDPGFESI 532
Sc ------------------------------------TYKAKNYVLAD------------SVSQEE--EEI 538
Sp ------------------------------------EIEM--LLDQGL----------VPCSRE---E-- 539
Dm RL-----------SASKAESDMA----------------FDMLPGQVEQHLGKREHYKKLSQK------- 644
Hs SLYRKHCLEQGKQQLMPQEAGLAEEFLLTDNSAIWQTVEELDYLEVEAEAKM-ENLRAAVPGQPLALTAR 722
Mm SLYRKHCLEQGKQQLEPQEVDLAEEFLLTDSSAMWQTVEELGCLDVEAEAKM-EN---SGLSQPLVLPAA 700
Xl DIYAEYSKNKTKDGCLSQHSTIPEEFMLTDDNSMWQKVEELSRTEMDLSSEVPATAKAKKRNNSSEHPVK 691
Gg RLYRQHCLQRGQEQ-PPEEAGVEDELMVLEGSSMWQKVEELSQLELDME-RP-GRAEQSQMQDEDGLPEL 135
   ●●●

Nc VDDRLRMDVDHK---------------YFKLPHKDGPNARCVNPMAKGYLPYFEKGILSSEYP---YAKE 640
Pp RAEDLK---NFT---------------FVKVPHPDGPSARVTNCMTKSCLGFFEKGFLNSQYP---LAKD 581
Sc RMNNLGLQCTGV---------------LFKVPHPNGPTFNCTNLLTKSYNHFFEKGVLKSESE---LAHQ 590
Sp -DTKLDYN-NYI---------------FFKVPHKDGPEARCCSPLSKSYHAYFEEGILQSDYE---VAKK 588
Hs GGPK-DTQPSYHHGNGPYNDVDIPGCWFFKLPHKDGNSCNVGSPFAKDFLPKMEDGTLQAG-PGGASGPR 790
Mm CAPK-SSQPTYHHGNGPYNDVNIPGCWFFKLPHKDGNNYNVGSPFAKDFLPKMEDGTLQAG-PGGASGPR 768
Xl LEMEFDSLPDNHHGNSPCGDVNVSGCWFYKLPHKDGNANNVGSPFAKDFLPKMEDGTLQAS-TGDSSATR 760
Gg VE-E-SSQPSFHHGNGPYNDVNIPGCWFFKLPHKDGNENNVGSPFAKDFLPRMEDGTLRAA-VGRTHGTR 202
Dm BQQRLETQ--YQGSGVWCNKVLDDCCFFLKLPHKNGPSFRVGNPLSKDFLNKFAENVLSSGDPSCQAAAR 710
                                * **  *          *          *

Nc ALEMNASCSYWI ARERIKNQMVVYEDQLPPS-QRFVNKDADSNTPIGGF--VLPQVI  GTITRRAVER 707
Pp ALQMAVASSYWT SRERIMNQFVV--------FE-------------DDMGYILPQII  GTITRRAVEN 630
Sc ALQINSSGSYWM ARERIQSQFVVPNCKFPNEFQSLSAKSSLNNEKTNDLAIIIPKIV  GTITRRAVEN 660
Sp ALEMSASCSYWS ARDRIRSQMVVWDKD----------AELGVPSSVDGFGIILPCII  GTVTRRAVEN 648
Dm VIDIARMMSYWRNNRDRIMGQMVVWLDS--QQLPNEFTGEK--CQP-IAYGAICPQVVACGTLTRRAMEP 775
Hs ALEINKMISFWRNAHKRISSQMVVWLPR--SALPRAVIRHPDYDEE-GLYGAILPQVVTAGTITRRAVEP 857
Mm ALEINKMISFWRNAHKRISSQMVVWLPR--SALPRVVTRHPAFDEE-GHYGAILPQVVTAGTITRRAVEP 835
Xl ALEINKMISFWRNAHKRISSQMVVWMKK--NELHRTITRDPEFDEE-NKYGAILAQVVSAGTITRRAVEP 827
Gg ALEINKMVSFWRNAHKRVSSQVVVWLKK--GELPRAVTRHPAYSEE-EDYGAILPQVVTAGTITRRAVEP 269
           * *     *   * **                                    ** **** *

Nc TWLTASNAKK RVGSELKAMVRAPPGYV VGADVDSEELWIASVVGDATFK-LHGGNAIGFMTLEGTKSQ 776
Pp TWLTASNAKK RLGSELKSLIEAPKGYC VGADVDSEELWIASLIGDSVFK-IHGGTAIGWMTLEGTKNE 699
Sc TWLTASNAKA RIGSELKTQVKAPPGYC VGADVDSEELWIASLVGDSIFN-VHGGTAIGWMCLEGTKNE 729
Sp TWLTASNSKK RLGSELKAMIRAPDGYT VGADVDSEELWIVALMGDSQFR-LHGATALGMMTLEGKKSE 717
Dm TWMTASNSRPDRLGSELRSMVQAPPGYRLVGADVDSQELWIASVLGDAYACGEHGATPLGWMTLSGSKSN 845
Hs TWLTASNARPDRVGSELKAMVQAPPGYTLVGADVDSQELWIAAVLGDAHFAGMHGCTAFGWMTLQGRKSR 927
Mm TWLTASNARPDRVGSELKAMVQAPTGYVLVGADVDSQELWIAAVLGDAHLAGMHGCTAFGWMTLQGRKSR 905
Xl TWLTASNARADRVGSELKAMVQVPPGYHLIGADVDSQELWIAAILGEAHFAGIHGCTAFGWMTLQGKKSS 897
Gg TWLTASNARADRVGSELKAMVQVPPGYSLVGADVDSQELWIAAVLGEAHFAGMHGCTAFGWMTLQGKKSD 339
   ** ****    * ****      * **   ****** ****            **    * * * * *

Nc GTDLHSRTAS  GITRNDAKVFNYGRIYGAGLKFASQLLRQFNPSLTEAETTAIATK YDATKGAK---- 843
Pp GTDLHSKTAK  GISRNEAKIFNYGRIYGAGIKFTTTLLKKFNPALSDAEAKATANA YTATKG-I---- 765
Sc GTDLHTKTAQ  GCSRNEAKIFNYGRIYGAGAKFASQLLKRFNPSLTDEETKKIANK YENTKG-K---- 795
Sp GTDLHSKTAA  GVSRDSAKVFNYGRLYGAGLKHTTLLLMQMNPTLKTAEAKELAKK YASTKGVK---- 784
Dm GSDMHSITAKAVGISRDHAKVINYARIYGAGQLFAETLLRQFNPTFSASEAKAKAMKMFSITKGKRVYRL 915
Hs GTDLHSKTATTVGISREHAKIFNYGRIYGAGQPFAERLLMQFNHRLTQQEAAEKAQQMYAATKGLRWYRL 997
Mm GTDLHSKTAATVRIHREHAKIFNYGRIYGAGQSFAERLLMQFNHRLTRQEAAEKAQQMYAVTKGLRRYRL 975
Xl GTDLHSKTASTVGISREHAKVFNYGRIYGAGQPFAERLLMQFNHRLTQEQAAEKAKQMYAVTKGIRRYIL 967
Gg GTDLHSKTAATVGISREHAKVFNYGRIYGAGQPFAERLLMQFNHRLTQQQAREKAQQMYAVTKGIRRFHL 409
   * * *  **      *  ** *** * ****      **   *           *   *  ***
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```
                      G
Nc ------------------------------------TNR---K---SLYKRPFWRGGTESFVFNMLEEFA  870
Pp ------------------------------------SGRYD-KK-------SIWYGGSESIIFNRLEAIA  790
Sc ------------------------------------TKRSKLFK-------KFWYGGSESILFNKLESIA  821
Sp ------------------------------------SKMSKRLQEMGLPKLTFWSQGTESFVFNKLEAMA  817
Dm R-E-E-FHDELEDRAYSSYEASRLAIQRNRTLA-------------EVFHRPKWQGGTESAMFNRLEEIA  969
Hs SDEGEWLVRELN-LPVDRTEGGWISLQDLRKVQRETARKSQWKK-WEVVAERAWKGGTESEMFNKLESIA 1065
Mm SADGEWLVKQLN-LPVDRTEDGWVSLQDLRMIRREASRKSRWKK-WEVASERAWTGGTESEMFNKLESIA 1043
Xl SKEGEWLVEELG-ISVERGEENSVNLQDLRKIQKDATKRSRRK--WNLVSRRIWTGGTESQMFNKLETIA 1034
Gg SEEGEWLVKELE-LAVDKAEDGTVSAQDVQKIQREAMRKSRRKKKWDVVAHRMWAGGTESEMFNKLESIA  478
                                                        *  * **  ** **  *

Nc EQERPRTPVLGAGITEALMSRWVSKGG--FLTSRINW[redacted]QSSGVDYLHL[redacted]IIAMDYLTRRFNLACR[redacted]AIT  938
Pp EMAHPKTPVLGAGITAPLQKANLSTNN--FLTSRINW[redacted]QSSGVDYLHL[redacted]IISMDYLIKLFDIDAR[redacted]CIT  858
Sc EQETPKTPVLGCGITYSLMKKNLRANS--FLPSRINW[redacted]QSSGVDYLHL[redacted]CCSMEYIIKKYNLEAR[redacted]CIS  889
Sp QLPSPRTPVLDAGIQQALSSKNLSKNS--FMTSRVNW[redacted]QSSAVDYLHL[redacted]LVSMNHLIKKYYLEAR[redacted]SLT  885
Dm TGSQPRTPFLGGRLSRALEADTGPEQEQRFLPTRINWVVQSGAVDFLHLMLVSMRWLMGS---HVRFCLS 1036
Hs TSDIPRTPVLGCCISRALEPSAVQEE---FMTSRVNWVVQSSAVDYLHLMLVAMKWLFEEFAIDGRFCIS 1132
Mm MSDTPRTPVLGCCISRALEPSVVQGE---FITSRVNWVVQSSAVDYLHLMLVAMKWLFEEFAIDGRFCIS 1110
Xl MSPSPKTPVLGCRISRALEPTAVKGE---FITSRVNWVVQSSAVDYLHLMLVAMKWLFEAYDIDGRFCIS 1101
Gg LSASPQTPVLGCHISRALEPAVAKGE---FLTSRVNWVVQSSAVDYLHLMLVSMKWLFEEYDINGRFCIS  545
       * ** *       *           *  ** *** *** ** ***    *  *        *

    [redacted]
Nc VHDEIRYLAEEPD[redacted]YRVAMALQIANL[redacted]TRVMFA[redacted]QVGIQDLPQSCAFFSAVDIDHVLRKEVDMDCITPSN 1008
Pp VHDEIRYLVKEED[redacted]FRAAYALQISNL[redacted]TRAMFC[redacted]QLGINEVPQSCAFFSAVDLDFVLRKEVDLDCVTPSN  928
Sc IHDEIRFLVSEKD[redacted]YRAAMALQISNI[redacted]TRAMFC[redacted]QMGINELPQNCAFFSQVDIDSVIRKEVNMDCITPSN  959
Sp VHDEVRYLSSDKD[redacted]YRVAFALQVANL[redacted]TRAFFC[redacted]RLGINELPQSVAFFSSVDIDHVLRKDVKMDCVTPSN  955
Dm FHDELRYLVKEELSPKAALAMHITNLMTRSFCVSRIGLQDLPMSVAFFSSVEVDTVLRKECTMDCKTPSN 1106
Hs IHDEVRYLVREEDRYRAALALQITNLLTRCMFAYKLGLNDLPQSVAFFSAVDIDRCLRKEVTMDCKTPSN 1202
Mm IHDEVRYLVREEDRYRAALALQITNLLTRCMFAYKLGLNDLPQSVAFFSAVDIDQCLRKEVTMDCKTPSN 1180
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Gg IHDEVRYLVQEQDRYRAALALQITNLLTRCMFAYKLGLQDLPQSVAFFSAVDIDRCLRKEVTMNCATPSN  615
    *** * *         * *    *  **       *    *   ****    *  ***     * ****
    Degenerate 3’primer (4)    Degenerate 3’primer (2)

         H
Nc PIP------IAHGESIDIFQILEKGDDAKLDDSIVPQSQYAPRLENIPYTPRVPVMQRLRERAEAGDHQA 1072
Pp PDP------IPCGKSLDIYQLLQQ-EDIKGADFPRTMHLNDVHYRKRTPVIEMFDKAVDDKTRKFMVSLQ  981
Sc KTA------IPHGEALDINQLLDK-PNSKLGKPSLDIDSKVSQYAYNYREPVFEEYNKSYTPEFLKYFLA 1022
Sp KVP------IPPGEELTIESVLEKLEQSGQSLEPLEQIQCFVDVKATTSAEITEEDKKNIA--YLKAQAF 1017
Dm PHGLRIGYGIQPGQSLSVAEAIEKAGGNDVSQWDWIKKS                                1145
Hs PTGMERRYGIPQGEALDIYQIIELTKGSLEKRSQPGP                                  1239
Mm PTGMERRYGIPQGEALDIYQIIELTKGSLEKRKPAWTLALSGGSVFAPVELYWSGAGS            1238
Xl PNGMEKRYGIPQGEALDIYQILKVTKGVL                                          1200
Gg PTGMEKKYGIPRGEALDIYQIIEITKGSLEKK                                        647
            *  *

Nc FLRFIRAQITNS--DEELKRIIAETRYSDPYGA-FSLASNGRVSGNPHQRHAAVHASTKTAAAPSKPSIA 1139
Pp IAQDKTEFTKWKRSRGELIVH                                                  1012
Sc MQVQSDKRDVNRLEDEYLRECTSKEYARDGNTAEYSLLDYIKDVEKGKR--------TKVRIMGSNFLDG 1084
Sp Y*                                                                     1018
Dm
Hs
Mm
Xl
Gg
```

```
Nc SRFDSVSQASRIKS-VAAGSDEPTIRATKAQGKAMA-KASGTKLAASTKDTVLNVTIKKKVAAPEMAAVP  1207
Pp
Sc TKNAKADQ--RIRLPV-NMPDYPTLHKI-ANDSAIPEKQLLENRRK--KENRIDDENKKKLTRKKNTT-P 1147
Sp
Dm
Hs
Mm
Xl
Gg


Nc STSSESKSKASATTSTTTTENATASPSSSSNVDAKKTTSKTKPTHKK-ETEGEPFPSLDDPVIAARLEAV 1276
Pp
Sc M---ERKYKRVYGGRKAF-E-------------AFYECAN-KPLDYTLETEKQFFNIPIDGVIDDVLNDK 1199
Sp
Dm
Hs
Mm
Xl
Gg


Nc S----KTSPGTRASVAA-KLDALASFCHASCCGC                                     1305
Pp
Sc SNYKKKPSQARTASSSPIRKTAKAVHSKKLPARKSSTTNRNLVELERDITISREY                1254
Sp
Dm
Hs
Mm
Xl
Gg
```

2.3.3 Transcription of *mip-1*

Northern blots of the total RNA from 10- to 12-hr old mycelia of *N. crassa* were examined for the presence of *mip-1* transcripts as described in Materials and Methods. A 4.5-kb mRNA was recognized by hybridization with a radioactively-labelled 3.3-kb *Cla*I DNA fragment originating from the internal region of *mip-1* (Figure 2-7). Thus, the transcript has combined 5' and 3' extensions of approximately 600 bp relative to the *mip-1* ORF.

2.3.4 Map location of *mip-1*

The chromosomal location of the *mip-1* gene was found by restriction fragment length polymorphism (RFLP) mapping using genomic DNAs from the strains generated by Metzenberg *et al.* (1984). The segregation of the RFLPs was detected by hybridization of Southern blots of the *Eco*RI-cut DNAs with probe generated from intact X25:10C cosmid, which includes the complete *mip-1* gene. On the basis of the RFLP map compiled by Nelson *et al.* (1998), the *mip-1* gene was found to be located on the right side of linkage group III, somewhere between *pro-1* and *ad-2* (Figure 2-8).

Because of its location, the *uvs-4* mutation in the LGIII was identified as a possible allele of *mip-1*. Thus, several PCR products, which cover the whole region of the *mip-1* gene, where generated and sequenced from the genomic DNA of *uvs-4* mutant. This comparison of the wild-type *mip-1* nucleotide sequence with the sequence of the *mip-1* DNA from *uvs-4* revealed no difference. Hence, it is unlikely that *uvs-4* is allelic to *mip-1*.

Figure 2-7. Detection of a transcript from *mip-1* by Northern blot hybridization. A: Total RNA from *N. crassa (N.c.)* mycelium run on an agarose gel. The lane containing RNA standard is labelled L. **B:** Northern blot of the *N.c.* lane from the gel in **A** hybridized to the radioactively labeled 3.3 kb *ClaI* internal *mip-1* fragment of X25:10C.

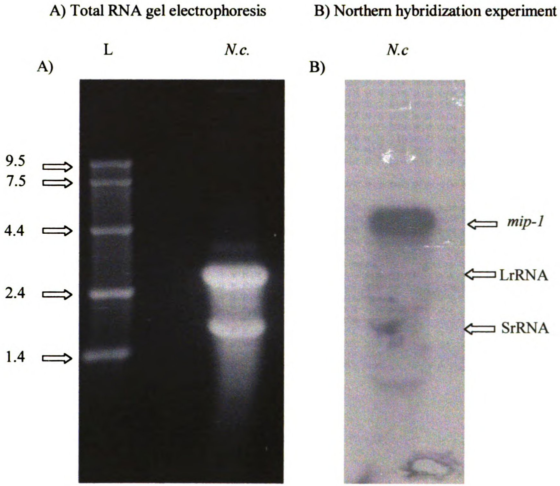

Figure 2-8. Restriction Fragment Length Polymorphysm (RFLP) mapping of the DNA polymerase-γ.

	50	51 52	53 54	55 56	57 58	59	60	61 62	63 64	65 66	67 68	69 70	71 72	73 74	75 76	77 78	79 80	81 82	83 84	85 86	87
	A	A|B	B|C	C|D	D|E	E	E	E|F	F|G	G|H	H|I	I|J	J|K	K|L	L|M	M|N	N|O	O|P	P|Q	Q|R	R
	1	4|6	7|1	4|5	7|1	3	5	7|1	3|1	4|5	7|6	8|1	4|1	4|1	4|5	8|2	3|2	4|1	4|2	4|1	4
LZC1	O	O|O	O|O	O|M	O|O	O	M	M|M	O|M	M|M	O|O	M|M	M|O	O|O	O|M	M|-	M|O	O|M	O|O	O|M	M
AP9b.2	O	O|O	O|M	M|M	O|O	O	M	M|M	O|M	M|M	O|O	M|M	M|O	O|O	M|O	O|M	M|M	M|O	O|O	O|M	M
AP12f.2	O	O|O	O|O	M|M	O|O	O	M	M|M	O|M	M|M	O|O	M|M	M|O	O|O	M|O	O|M	M|M	M|O	O|O	O|M	M
X14:1F	O	O|O	O|O	M|M	O|O	O	-	-|M	O|M	M|M	O|O	M|M	M|O	O|O	M|O	O|M	M|M	M|O	O|O	O|M	M
ace-2	O	O|O	O|M	M|M	O|O	O	M	M|M	O|M	M|M	M|O	M|M	M|O	O|O	M|O	O|M	M|M	M|O	O|O	O|M	M
con-1	O	M|-	O|M	M|M	M|O	O	M	M|M	M|-	M|M	M|O	O|M	O|O	M|O	M|O	O|O	M|M	M|O	O|O	O|O	M
cyt-8	O	O|O	O|M	M|M	M|O	O	M	M|M	M|O	M|M	M|O	O|M	O|O	O|M	M|O	O|M	M|M	M|O	O|O	O|M	M
R15.4	O	O|O	O|M	M|M	M|O	O	M	M|M	M|M	M|M	-|O	-|M	M|O	O|M	M|O	O|O	O|M	M|O	O|O	O|M	M
AP31a.4	O	O|O	O|M	M|M	M|O	O	M	M|M	M|M	M|M	M|O	-|M	M|O	O|M	M|O	O|O	O|M	M|O	O|O	O|M	M
ser-1	O	O|O	O|M	-|M	-|O	O	M	M|M	M|M	M|M	M|O	M|M	M|O	O|M	M|O	O|M	M|M	M|O	O|O	O|M	M
krev-1	O	O|O	O|-	-|M	-|-	O	M	M|M	M|-	M|M	M|O	M|M	M|O	O|M	M|O	O|M	M|-	M|O	O|-	O|M	M
Cen III, crp-2	O	O|O	O|M	M|M	M|O	O	M	M|M	M|M	M|M	M|O	O|M	M|O	O|M	M|O	O|M	M|M	M|O	O|O	O|M	M
thi-4	O	O|O	O|M	M|M	O|O	O	M	M|M	M|M	M|M	M|-	O|M	M|O	O|M	M|O	O|M	M|M	M|O	O|O	O|M	M
pSK7-81C	O	O|O	O|M	M|M	O|O	O	M	M|M	M|M	M|M	M|O	O|M	M|O	O|M	M|O	O|M	M|M	M|O	O|O	O|M	M
pro-1	O	O|O	O|M	M|M	O|O	O	M	M|M	M|M	M|M	M|O	M|M	M|O	O|M	M|O	O|M	-|M	M|O	O|O	O|M	M
LZE5	O	O|O	O|M	O|M	O|O	O	M	M|M	O|M	M|M	M|-	M|M	M|O	O|M	M|O	O|M	M|M	M|O	O|O	O|M	M
RRK	O	O|O	O|M	-|M	-|-	O	M	M|M	O|-	M|M	M|O	M|M	M|O	O|M	M|O	O|M	M|-	M|O	O|O	O|M	M
LZE4	-	O|O	O|-	-|M	O|O	O	M	M|M	O|M	M|-	-|O	M|M	M|O	O|M	M|O	O|M	M|M	M|M	M|O	O|O	O
X25:10C(*mip-1*)	**O**	**O|O**	**-|O**	**M|M**	**O|O**	**O**	**M**	**M|M**	**O|M**	**M|M**	**O|O**	**O|M**	**M|O**	**O|O**	**M|O**	**O|M**	**M|M**	**M|M**	**O|O**	**O|M**	**M**
G22:1H	O	O|O	O|O	M|M	O|O	O	M	M|M	O|M	M|M	O|O	O|M	M|O	O|O	M|O	O|M	M|M	M|M	O|O	O|M	M
con-7	O	O|-	O|O	M|M	O|O	O	M	M|M	O|M	M|M	O|O	O|M	M|O	O|O	M|O	O|M	M|M	M|M	O|O	O|M	M
ad-2	O	O|O	O|O	M|M	O|O	O	M	M|M	O|M	M|M	O|O	M|M	M|O	O|O	M|O	O|M	M|M	M|M	O|O	O|M	M
trp-1	O	O|-	O|O	M|M	O|O	O	M	M|M	O|-	M|M	O|O	M|M	M|O	O|O	M|O	O|M	M|M	M|M	O|O	O|M	M
DB002	O	O|O	O|O	M|M	O|O	O	M	M|M	O|M	M|M	O|O	M|M	M|O	O|O	-|O	O|M	M|M	M|-	O|M	O|M	M
AP32a.2	M	O|M	M|O	M|M	O|-	O	M	M|M	O|M	-|M	O|O	M|M	M|O	O|O	M|O	M|M	M|M	M|O	-|O	O|M	M
32:2G	O	O|O	O|O	M|M	O|-	O	M	M|M	O|M	M|M	O|-	M|M	M|O	O|O	M|O	O|M	O|M	M|M	O|O	O|M	M
Fsr-45	M	O|M	O|O	O|O	M|O	M	O	M|M	O|M	M|O	O|M	O|M	O|O	M|O	O|O	M|O	O|O	M|M	O|O	M|M	O
00032	M	O|O	O|O	M|O	M|-	M	O	M|M	O|M	O|O	O|M	O|M	O|M	O|O	O|O	M|O	O|O	M|M	O|O	M|M	O
cat-3	M	O|M	O|O	O|O	M|O	M	O	M|O	M|M	M|O	O|M	O|M	O|M	M|O	O|O	M|O	M|O	M|M	O|O	M|M	O
Ncr-6	M	O|M	O|-	M|O	M|O	-	O	M|O	M|M	M|O	-|M	-|M	-|M	M|M	M|-	M|-	M|O	M|-	O|O	M|O	O
AP8f.1	M	O|M	O|O	M|O	M|O	M	O	M|O	M|M	M|O	O|M	O|M	O|O	M|O	O|O	M|O	O|O	M|M	O|O	O|M	O
AP12f.4	M	O|M	O|O	M|O	M|O	O	O	M|O	M|M	M|O	O|M	O|M	O|O	M|O	O|O	M|O	O|M	M|M	O|O	M|M	O
Tel IIIR	M	O|M	O|O	M|O	M|O	M	O	-|-	M|O	M|O	O|M	O|M	O|O	M|O	O|O	M|O	O|M	M|M	O|O	M|M	O

2.1 Summary and Conclusions

Duplication of any DNA genome is the result of many enzymes and other proteins. I have cloned and sequenced the DNA polymerase-γ gene (*mip-1*) from *N. crassa* using degenerate PCR primers designed from conserved regions of gamma DNA polymerase from other species. The *N. crassa mip-1* gene encodes a protein of 1305 amino-acids and which has a mitochondrial targeting element. The *N. crassa mip-1* gene was located on the right side of linkage group III between *pro-1* and *ad-2*. The *N. crassa* and *S. cerevisiae* polymerase-γ polypeptides have long C-terminal extensions that are not found in the homologous proteins from other species. The higher eukaryotic gamma DNA polymerases have 8 insertion sites totaling approximately 200 amino-acid residues. The gamma DNA polymerases can be divided in two evolutionary groups on basis of the presence and absence of inserts A to H. At this time, it is not known whether or not the γ-DNA polymerase that has been cloned is essential for the replication of the mtDNA in *N. crassa*. However, the role of the protein in the maintenance of the mitochondrial genome of this fungus now can be analyzed through targeted mutagenesis and observation of the effects of the mutations in homokaryons extracted from sheltering heterokaryons.

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